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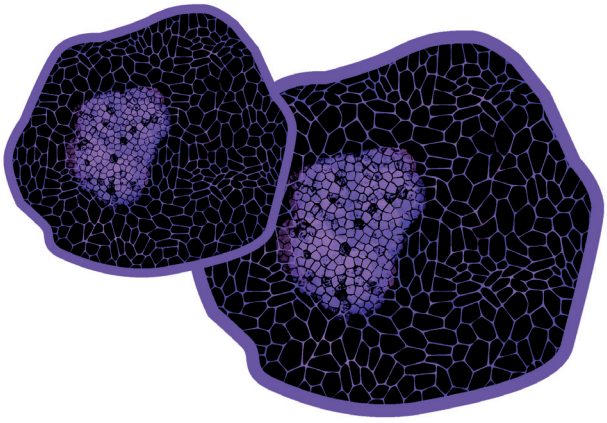
KATARIINA MAANINKA

# Atheroinflammatory Properties of LDL and HDL Particles Modified by Human Mast Cell Neutral Proteases



WIHURI RESEARCH INSTITUTE AND
DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

# ATHEROINFLAMMATORY PROPERTIES OF LDL AND HDL PARTICLES MODIFIED BY HUMAN MAST CELL NEUTRAL PROTEASES

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ACADEMIC DISSERTATION
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*If the path before you is clear, you're probably on someone else's.*

*-Joseph Campbell*

*To My Family*

# TABLE OF CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications.

**I** **Maaninka K**, Lappalainen J, Kovanen PT. Human mast cells arise from a common circulating progenitor. J Allergy Clin Immunol 2013;132:463-469 e463.

**II** Nguyen SD, **Maaninka K**, Lappalainen J, Nurmi K, Metso J, Öörni K, Navab M, Fogelman AM, Jauhiainen M, Lee-Rueckert M, Kovanen PT. Carboxyl-terminal cleavage of apolipoprotein A-I by human mast cell chymase impairs its anti-inflammatory properties. Arterioscler Thromb Vasc Biol 2016;36:274-284.

**III** **Maaninka K**, Nguyen SD, Mäyränpää MI, Plihtari R, Rajamäki K, Lindsberg PJ, Kovanen PT, Öörni K. Human mast cell neutral proteases generate modified LDL particles with increased proteoglycan binding. Atherosclerosis 2018, https://doi.org/10.1016/j.atherosclerosis.2018.04.016

AUTHOR'S CONTRIBUTION TO THE ARTICLES

**I** Author participated in the study design, conducted all the experiments and data analyses with the exception of qPCR primer design and statistical analyses, interpreted the results and wrote the manuscript.

**II** Author participated in the study design, conducted mast cell differentiation, culture, and activation, as well as protease activity assays, participated in animal experiments, data analyses, interpretation of the results, and writing the manuscript.

**III** Author participated in the study design and conducted all the experiments with the exception of immunofluorescence staining, circular dichroism, coronary RNA isolation, and some of the electron microscopy, conducted all the data analyses, interpreted the results, and wrote the manuscript.

The publications are referred to in the text by their Roman numerals. The original publications are reproduced with the permission of the copyright holders.

# ABBREVIATIONS

Abbreviations used more than once are included in the list.

| | |
|---|---|
| (A-I)rHDL | apolipoprotein A-I –containing reconstituted high-density lipoprotein |
| ABC | ATP-binding cassette |
| ACVD | atherosclerotic cardiovascular disease |
| apo | apolipoprotein |
| CD | circular dichroism |
| CE | cholesteryl ester |
| CEH | cholesteryl ester hydrolase |
| CEPT | cholesteryl ester transfer protein |
| CPA | carboxypeptidase A |
| CS | chondroitin sulfate |
| EC | endothelial cell |
| ECM | extracellular matrix |
| FcεRI | Fc epsilon receptor I/high-affinity receptor for immunoglobulin E |
| FH | familial hypercholesterolemia |
| GAG | glycosaminoglycan |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| HCAEC | human coronary artery endothelial cell |
| HDL | high-density lipoprotein |
| HDL-C | high-density lipoprotein cholesterol |
| 4-HNE | 4-hydroxynonenal |
| ICAM-1 | intercellular adhesion molecule 1 |
| IDL | intermediate-density lipoprotein |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| KITLG | kit ligand |
| LAL | lysosomal acid lipase |
| LCAT | lecithin:cholesterol acyltransferase |
| LDL | low-density lipoprotein |
| LDL-C | low-density lipoprotein cholesterol |
| LDLR | low-density lipoprotein receptor |
| Lp(a) | lipoprotein(a) |
| LPS | lipopolysaccharide |
| MC | mast cell |
| $MC_C$ | mast cell containing only chymase |
| $MC_T$ | mast cell containing only tryptase |
| $MC_{TC}$ | mast cell containing tryptase and chymase |
| MCP-1 | monocyte chemotactic protein -1 |
| MDA | malodialdehyde |
| MMP | matrix metalloproteinase |
| NF-κb | nuclear factor-κb |
| oxLDL | oxidized low-density lipoprotein |
| PG | proteoglycan |
| RCT | reverse cholesterol transport |
| rHDL | reconstituted high-density lipoprotein |
| sSMase | secretory sphingomyelinase |
| SMC | smooth muscle cell |
| $sPLA_2$ | secretory phospholipase $A_2$ |
| SR | scavenger receptor |
| TAG | triacylglycerol |
| TG | triglyceride |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| VCAM-1 | vascular cell adhesion molecule 1 |
| VLDL | very low-density lipoprotein |

# ABSTRACT

Atherosclerosis is a slowly progressing disease characterized by the development of inflamed fatty deposits called atherosclerotic lesions or plaques within the innermost layer of the arterial wall, the intima. First subclinical atheromatous changes within the arterial intima begin early in childhood as a response to cholesterol accumulation and serve as precursors for more advanced lesions that develop during the following decades through complex series of cellular events. During the degenerative process of lesion formation, the affected arteries gradually lose their normal structure and function, and may become narrowed by the growing plaques, a process leading to impeded blood flow to end-organ. Eventually multiple subclinical cellular events may result in the development of unstable, vulnerable plaques that are prone to rupture culminating in the life threatening clinical manifestation of myocardial infarction or ischemic stroke.

Atherosclerosis is strongly associated with lifestyle and age. Enhanced plasma level of low-density lipoprotein (LDL) is considered a definitive risk factor of atherosclerosis, emphasizing the crucial role of LDL in the process of lesion development. Circulating LDL particles are the major carriers of cholesterol to the arterial intima and their retention by subendothelial proteoglycans and subsequent modification within the intima drives intramural cholesterol accumulation and ensuing formation of atherosclerotic lesions. Another lipoprotein playing a central, yet opposite role in atherogenesis is the high density lipoprotein (HDL). HDL and its principal protein component, apolipoprotein A (apoA)-I, act against atherosclerotic lesion development by removing cholesterol from the arterial wall and delivering it to liver for excretion. In addition to this process referred to as reverse cholesterol transport, HDL and apoA-I possess various anti-inflammatory properties by which they may hinder the inflammatory processes involved in the plaque development, and thus to protect from the disease progression. However, similar to LDL, HDL within the arterial intima is subjected to various structural and compositional modifications that impair its normal physiological function leading to generation of HDL particles with reduced antiatherogenic properties.

LDL and HDL modifications within the arterial intima can be induced by extracellular enzymes released from cells present in the intima. MCs are tissue-dwelling effector cells of innate and adaptive immunity that differentiate from committed circulating progenitor cells and are present in increased numbers in atherosclerotic lesions. Being capable of releasing large amounts of various neutral proteases upon activation, MCs are potential source of LDL and HDL proteolyzing enzymes. The aim of this thesis was to investigate the ability of various human MC neutral proteases to cleave the protein moieties of LDL and HDL particles, apoB-100 and apoA-I, respectively and thus to contribute to

the pathogenic processes of LDL accumulation and inflammation both essential in development of atherosclerotic lesions.

In the present study, human MCs expressing four tryptase isoforms, chymase, carboxypeptidase A3, cathepsin G, and granzyme B are described. Of these, chymase and cathepsin G efficiently proteolyzed apoA-I and apoB-100, respectively generating C-terminally truncated apolipoproteins. Proteolysis by chymase resulted in reduced anti-inflammatory properties of apoA-I and impaired the ability of apoA-I to induce cholesterol efflux from macrophage foam cells. Furthermore, proteolysis of apoB-100 by cathepsin G induced formation of fused LDL particles with increased binding to human aortic proteoglycans and atherosclerotic lesions of human carotid arteries. Taken together, the data presented in this thesis propose novel mechanisms by which human MC neutral proteases may promote lipid accumulation and inflammation within the arterial wall and thus promote lesion development and progression.

# TIIVISTELMÄ

Ateroskleroosi (valtimonkovettumatauti) on suurten verisuonien eli valtimoiden sairaus, jossa valtimoiden seinämään kertyy rasvaa ja tulehdussoluja. Valtimoihin kerääntyvä rasva on pääosin kolesterolia, joka on peräisin low-density lipoproteiini (LDL)-hiukkasista. LDL on elimistön kemiallinen yhdiste, joka kuljettaa kolesterolia veren mukana sen synteesipaikasta maksasta muualle elimistöön, jossa solut käyttävät kolesterolia muun muassa kalvojensa rakennusaineeksi. Valtimoiden seinämään kolesteroli päätyy verenkierrosta kun LDL-hiukkanen läpäisee valtimon sisäpintaa verhoavan solukerroksen. Valtimon seinämän sisimmässä kerroksessa LDL jää kuitenkin kiinni seinämän soluväliaineen muodostamaan tiheään verkkoon, mikä estää hiukkasen poistumisen seinämästä. Seurauksena LDL-hiukkaset ja niiden sisältämä kolesteroli kerääntyvät valtimon seinämään.

Valtimon seinämässä LDL-hiukkasten tavanomainen rakenne ja koostumus ovat alttiita muokkaantumiselle, mikä edelleen vaikeuttaa niiden ulospääsyä seinämästä ja kiihdyttää LDL-kolesterolin kertymistä seinämään. Seurauksena syntyy krooninen tulehdusreaktio, joka johtaa vähitellen yhä suurempien kolesterolikertymien kehittymiseen. Koska kolesterolin kertyminen alkaa jo nuoruudessa ja etenee hitaasti vuosikymmenien kuluessa, ateroskleroosi kehittyy salakavalasti eikä yleensä aiheuta oireita ennen kuin kolesterolikertymät, niin kutsutut ateroskleroottiset plakit, kasvavat niin suuriksi, että ne alkavat ahtauttaa valtimoa haitaten hapekkaan veren kulkeutumista kohdekudokseen. Koska suurten tulehduksellisten plakkien pinta on pehmeää ja haurasta kudosta, se on altis repeytymään, jolloin paikalle muodostuu verihyytymä. Seurauksena on tavallisimmin joko aivo- tai sydäninfarkti.

Valtimonkovettumataudin kehittyminen on yhteydessä riskitekijöihin, joista useimmat liittyvät elintapoihin. Korkea veren LDL pitoisuus lisää valtimoon kerääntyvän kolesterolin määrää ja täten riskiä sairastua valtimonkovettumatautiin. Sen sijaan high-density lipoproteiini (HDL)-hiukkasten pitoisuus veressä on kääntäen verrannollinen sairastumisriskiin. HDL:n suojaavan vaikutuksen ajatellaan perustuvan sen ja sen pääasiallisen proteiiniosan, apolipoproteiini (apo) A-I:n kykyyn poistaa kolesterolia valtimon seinämästä ja hillitä tulehdusreaktioita ateroskleroosin kehittymisen kannalta merkittävissä soluissa, minkä seurauksena ateroskleroottisten plakkien kehittyminen hidastuu. Ateroskleroosin kehittymisessä avainasemassa ovatkin siis kolesterolin kuljetushiukkaset LDL ja HDL, eikä niinkään kolesterolipitoisuus itsessään. Kuten LDL, myös HDL on kuitenkin altis muokkaantumaan valtimon seinämässä siten, että sen kyky suojata ateroskleroosilta heikkenee.

Syöttösolu on eräs ateroskleroottisissa plakeissa esiintyvä tulehdussolutyyppi, jonka on osoitettu osallistuvan ateroskleroosin kehittymiseen vapauttamalla aktivoiduttuaan proteiineja pilkkovia entsyymejä, niin kutsuttuja neutraaleja proteaaseja. Ateroskleroottisista plakeista löydetyt syöttösolut voidaan jakaa alatyyppeihin sen perusteella, mitä neutraaleja proteaaseja ne ilmentävät. Koska eri neutraaliproteaaseilla on kyky pilkkoa eri proteiineja, syöttösolujen alatyypeillä on mahdollisesti kliinistä merkitystä valtimonkovettumataudin kannalta.

Tässä väitöskirjatyössä tutkittiin ihmisen verenkierrosta erilaistettujen syöttösolujen kykyä ilmentää eri neutraaliproteaaseja ja näiden proteaasien kykyä pilkkoa LDL- ja HDL-hiukkasten proteiiniosia, apoB-100:a ja apoA-I:a sekä tästä seuraavia mahdollisia ateroskleroosin kehittymisen kannalta merkityksellisiä vaikutuksia. Tutkimuksessa kuvataan ensimmäistä kertaa syöttösolutyyppi, joka ilmentää neutraaleihin proteaaseihin kuuluvia neljää erilaista tryptaasia sekä kymaasia, karboksipeptidaasi A3:a, katepsiin G:a ja grantsyymi B:a. Lisäksi tutkimuksen tulokset osoittavat, että näistä kymaasi pilkkoo HDL-hiukkasen apoA-I:a siten, että sen kyky poistaa valtimon seinämään kerääntynyttä kolesterolia sekä sen kyky estää valtimonkovettumataudin kannalta merkityksellisten solujen tulehduksellista aktivoitumista heikkenee. Tutkimuksessa havaittiin lisäksi, että katepsiini G pilkkoo LDL-hiukkasen apoB-100:a siten, että LDL-hiukkasten koko kasvaa ja niiden vuorovaikutus valtimon seinämän kanssa lisääntyy. Koska LDL-hiukkasten ja valtimon seinämän välinen vuorovaikutus on tärkeä ateroskleroosia edistävä tekijä, sen taustalla olevien mekanismien tunnistaminen tarjoaa mahdollisuuden uudentyyppisten, LDL-hiukkasten ja valtimonseinämän fysikaalisen vuorovaikutuksen estämiseen perustuvien hoitomuotojen kehittämiseen ateroskleroosin hoitoon. Lisäksi, koska apoA-I:a proteolyyttinen pilkkoutuminen heikentää sen terapeuttista potentiaalia, tutkimuksen tulokset nostavat esille proteaasien aiheuttamalle pilkkoutumiselle vastustuskykyisten apoA-I:a matkivien molekyylien mahdollisen hyödyn tämän maailmanlaajuisesti merkityksellisen taudin hoidossa.

# INTRODUCTION

Atherosclerosis is a degenerative disease of large- and medium-sized arteries characterized by focal development of cholesterol-rich, inflamed deposits, called atherosclerotic lesions or plaques, along the arterial tree. Development of atherosclerotic plaques causes hardening (loss of elasticity) of the artery and may lead to reduced blood flow to end organ, such as heart muscle or brain. Atherosclerosis is strongly associated with lifestyle and has a number of well-determined risk factors. These include hypertension, smoking, obesity, diabetes, lack of exercise, family history, high low-density lipoprotein cholesterol (LDL-C), low high-density lipoprotein cholesterol (HDL-C), and high triglycerides (TGs) (Lusis 2000, Fruchart et al., 2004). First morphological signs of atherosclerosis appear early in the childhood, after which the disease progresses silently for decades. Almost all people are affected to some degree by the age of 65, however symptoms, if any, occur only after narrowing of an artery (stenosis) impedes blood flow to end-organ enough to induce them. Continuous lipid accumulation and heightened inflammatory status may ultimately result in rupture of the atherosclerotic plaque fostering a formation of a blood clot that completely blocks the artery. Such acute event is the underlying cause of myocardial infarction and ischemic stroke, the leading causes of death globally (Benjamin et al., 2017).

The term '*atherosclerosis*' is apparently first introduced by Felix Marchand in 1904 and derives from the Greek "athera," meaning gruel, or wax, corresponding to the porridge-like necrotic core area at the base of the atherosclerotic plaque, and "sclerosis" meaning hardening, referring to the fibrous cap of the plaque's luminal edge. The first to demonstrate the role of cholesterol in the development of atherosclerosis was the Russian scientist Nikolai N. Anitschkov (1885-1964), whose classical experiments showed that feeding cholesterol to rabbits caused atheromatous changes in the arterial wall similar to what was found in humans [reviewed in (Konstantinov et al., 2006)]. Yet, an avalanche of research in cholesterol-induced atherosclerosis was produced by the identification in the mid-1950s that LDL-C is responsible for the rapid progression of atherosclerosis in humans (Gofman&Lindgren 1950). Nowadays it is widely accepted that the uptake into the vessel wall of LDL followed by LDL retention and modification within the arterial wall induce inflammatory responses that promote development of atherosclerotic lesions. Similarly, it has become obvious that HDL, the counter actor of LDL, protects from atherosclerosis by removing cholesterol from the arterial wall and by possessing various anti-inflammatory properties towards vascular and inflammatory cells.

Within the same decade that the role of LDL-C in atherosclerosis was elucidated, a tissue-dwelling inflammatory cell of hematopoietic origin, the mast cell (MC), was

first suggested to be involved in the susceptibility to experimental atherosclerosis. The hypothesis was proposed by Constantinides in 1953 based on his studies (Constantinides 1953, Constantinides et al., 1953) suggesting that MCs, via heparin proteoglycan (PG) present in their granules, could be protective from atherosclerosis. Emerging number of studies in the following decades, however, gradually shifted the concept towards a pro-atherogenic role for MCs, as MC numbers in the different layers of the arterial wall were found to be increased with the progression of atherosclerosis (Atkinson et al., 1994, Kaartinen et al., 1994b, Kovanen et al., 1995, Jeziorska et al., 1997).

MCs were originally discovered in 1877 by a medical student Paul Ehrlich, the later German scientist, who received the Nobel Prize in 1908 for his contributions to immunology [reviewed in (Crivellato et al., 2003)]. Ehrlich noticed that cells stained with aniline blue were full of cytoplasmic granules that had turned from a blue to a reddish color, a phenomenon referred to as metachromasia. Ehrlich believed that the granules were the result of overfeeding, and named the cells Mastzellen based on "mästen" in German, which refers to feeding. To date, MCs are best known as key effector cells mediating the immunoglobulin E (IgE)-dependent allergic reactions (Galli&Tsai 2012), however, potential roles for MCs at various stages of atherosclerosis have also been established (Kovanen 2007a, Bot et al., 2015).

Human MCs have been traditionally classified into two major subtypes distinct in their granule protease composition. Originally the subtypes were found to differentially store two MC-specific neutral proteases, chymase and tryptase within their granules, and were thus termed as the $MC_T$ for an MC containing only tryptase and $MC_{TC}$ for an MC containing both tryptase and chymase (Irani et al., 1986, Irani et al., 1989). Both subtypes have been identified in atherosclerotic lesions (Kaartinen et al., 1994a, b), however, it has been a matter of debate, whether the phenotypes of these subtypes are precommited or whether they represent adaptation to different functional properties. Since many of the effector functions of MCs are attributed to their neutral proteases (Kovanen 2007b), which MCs release upon activation to extracellular microenvironment, the existence of tissue MCs with different combinations of proteases having different peptide and protein targets, may be of clinical importance. The aim of this study was to clarify the concept of the heterogeneity and plasticity of human MC phenotypes by investigating protease expression in cultured human MCs and to investigate the ability of the expressed neutral proteases to contribute to atherogenesis via proteolyzing the major protein moieties of LDL and HDL particles.

# REVIEW OF THE LITERATURE

## 1 Atherosclerosis and plasma lipoproteins

It has been over a century since the concept of atherosclerosis as a cholesterol-driven disease was introduced [reviewed in (Konstantinov et al., 2006)]. During the following decades, it has been discovered that the key sources of cholesterol in the atherosclerotic lesions are apolipoprotein B (apoB)-containing lipoproteins from plasma. There is now vast evidence—from epidemiology, genetics (including Mendelian randomization studies), experimental models, and clinical trials—to prove that the role of cholesterol-rich apoB-containing lipoproteins in the pathogenesis of atherosclerosis is fundamental and causative (Ference et al., 2017). Indeed, the direct link between plasma cholesterol of apoB-containing lipoproteins and atherosclerosis is emphasized by the success of cholesterol-lowering drugs in the treatment of atherosclerosis.

The apoB-containing lipoproteins comprise various particles that can be separated by ultracentrifugation into different subclasses based on their buoyant densities. These subclasses are in the order of decreasing size and increasing density, chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and LDL (Figure 1). Of these, LDL is the principal driver of atherosclerotic plaque development. A non-apoB-containing lipoprotein, HDL, plays also a crucial, yet opposing, role in atherosclerosis. HDL is regarded as anti-atherogenic, mainly because of its role in cellular cholesterol extraction (Navab et al., 2011), which hinders cholesterol accumulation within arterial walls. It has become obvious, however, that the relationship between HDL and atherosclerosis is more complex than that between LDL and atherosclerosis (Rohatgi 2015). The following sections describe the structural and compositional characteristics of LDL and HDL, the two lipoprotein subclasses most relevant in the pathobiology of atherosclerosis, as well as their roles in the development of atherosclerotic lesions, the process referred to as atherogenesis.

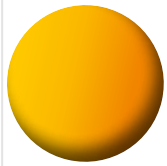
| | Chylomicron | VLDL | IDL | LDL | HDL |
|---|---|---|---|---|---|
| |  |  |  |  |  |
| Diameter (nm) | 1000 | 35–90 | 25–35 | 20–25 | 10 |
| Density (g/ml) | <0.95 | <1.006 | 1.006–1.019 | 1.019–1.063 | 1.063–1.210 |
| Average blood level (mM)* | N/A | 0.1 –0.7 | N/A | 2.6–3.9 | 1.3–2.0 |
| Source | Intestine | Liver | VLDL catabolism | IDL catabolism | Liver, intestine |
| TAG (%) | 86 | 55 | 31 | 6 | 4 |
| CE (%) | 3 | 12 | 23 | 42 | 12–20 |
| UC (%) | 2 | 7 | 7 | 8 | 4 |
| PL (%) | 7 | 18 | 22 | 22 | 25–30 |
| Protein (%) | 2 | 8 | 15 | 22 | 44–50 |
| Major apolipoproteins | ApoB-48<br>ApoC-I, -II, III<br>ApoE | ApoB-100<br>ApoC-I, -II, III<br>ApoE | ApoB-100<br>ApoE | ApoB-100 | ApoA-I, -II, -IV<br>ApoE |

***Figure 1. Plasma lipoproteins of various subclasses.*** *Human plasma lipoproteins are macromolecular complexes that transport hydrophobic lipids between tissues through the circulatory system. Mature plasma lipoproteins are spherical particles composed of a hydrophobic core formed by triacylglycerols and cholesteryl esters, and a monolayer of amphipathic phospholipids, unesterified cholesterol, and apolipoproteins. Different combinations of apolipoproteins and lipids produce lipoprotein particles with different densities that can be separated by ultracentrifugation into various subclasses. The major plasma lipoprotein subclasses in humans include, in the order of increasing densities and decreasing size, chylomicrons, VLDL, IDL, LDL, and HDL. Each lipoprotein subclass has a unique role determined by its site of origin, lipid composition, and apolipoprotein content. N/A, not available. The chylomicrons contain C-terminally truncated apoB, apoB-48, and carries dietary lipids from intestine to liver. The apoB-100-containing lipoprotein VLDL carries lipids of hepatic origin to extrahepatic tissues and gives rise to LDL via IDL through a metabolic cascade in the circulation. LDL is the major cholesterol carrier of human plasma and drives atherogenesis by promoting cholesterol accumulation within the arterial wall. HDL is an apoA-containing lipoprotein, which carries cholesterol from the extrahepatic tissues back to liver for excretion, and is thus regarded as anti-atherogenic. *Values vary depending on age, sex, and life style. (%) of dry weight. Data from (Chapman 1986, Havel&Kane 2001, Jonas 2002, Langsted et al., 2008). CE, cholesteryl ester; N/A, not available; PL, phospholipid; TAG, triacylglycerol; UC, unesterified cholesterol.*

## 1.1 Low-density lipoprotein

Human plasma LDL particles are spherical particles with the average size of 22 nm in diameters and density ranging from 1.019 to 1.063 g/ml (Lindgren et al., 1951, Havel et al., 1955, Hevonoja et al., 2000). The physiological role of LDL is to provide cholesterol for cells of extrahepatic tissues, which bind and internalize LDL via their plasma membrane receptors (Brown&Goldstein 1986). LDL is produced through a metabolic cascade in the circulation from VLDL, which again is synthesized in the liver (Robinson 1973). From the liver VLDL is secreted into the circulation, where endothelial lipoprotein lipase in the capillaries of muscle and adipose tissues releases free fatty acids from the triacylglycerols (TAGs) present in the VLDL core (Robinson 1973, Chapman et al., 2011). In addition, some of the surface lipids and apolipoproteins (C and E) of VLDL are transferred to HDL, in a process mediated by lipoprotein lipase and lipid transfer proteins in plasma (Tall 1995, Murdoch&Breckenridge 1996, Chapman et al., 2011). VLDL remnants remain in the blood and are converted first into IDLs and further into LDLs, in a process that involves hepatic lipase (Demant et al., 1988, Chapman et al., 2011). As a resultant particle of such metabolic cascade, LDL contains much less TAGs than VLDL, but, instead is very rich in cholesteryl esters (CEs). In addition, due to removal of apoCs and apoE from VLDL to HDL, LDL contains apoB-100 as virtually its sole apolipoprotein. The composition of LDL is portrayed in Figure 2.

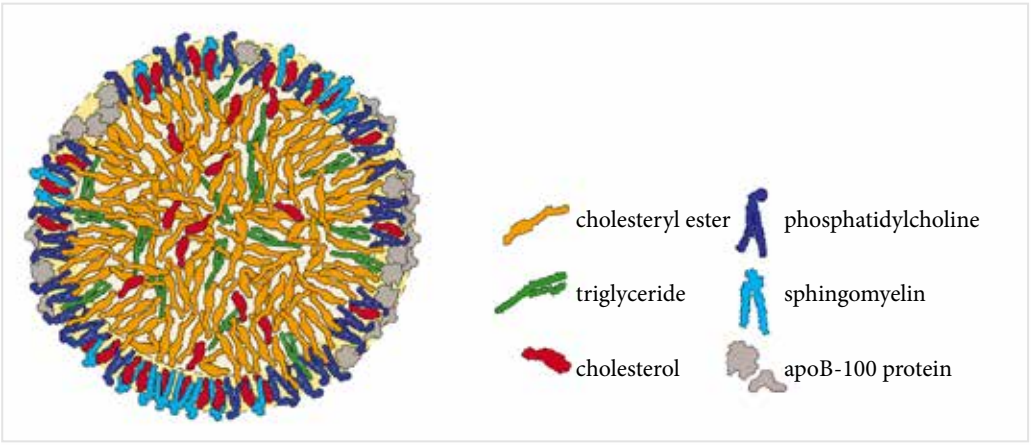



***Figure 2. Composition of LDL particle.*** *LDL is composed of a hydrophopic core of neutral lipids consisting of approximately 1600 molecules of cholesteryl esters and 170 molecules of triacylglycerols. The surface monolayer consists of about 700 molecules of phospholipids and a single copy of apoB-100. In addition, one LDL particle contains, on average, 600 molecules of unesterified cholesterol, of which approximately one third lies in the core and two thirds reside on the surface. The main phospholipid components of LDL are phosphatidylcholine and sphingomyelin, which constitute about 70% and 30% of the phospholipids, respectively. In addition, one LDL particle contains, on average, 80 molecules of lysophosphatidylcholine, 10 molecules of phosphatidylethanolamine, 7 molecules of diacylglycerol, molecules of ceramide, and some phosphatidylinositol molecules. Data from (Lund-Katz&Phillips 1986, Esterbauer et al., 1992, Hevonoja et al., 2000). Image modified from (Hevonoja et al., 2000) with the permission of the copyright holder.*

## 1.2 Apolipoprotein B-100

ApoB-100 is a large protein that envelopes the surface of pro-atherogenic lipoproteins of hepatic origin (VLDL, IDL, and LDL). In addition, lipoprotein(a) [Lp(a)], a unique lipoprotein contains apoB-100 to which apo(a), a member of the plasminogen gene family has been covalently attached (Anuurad et al., 2006). Consisting of 4536 amino acid residues (Chen et al., 1986, Knott et al., 1986) and with a molecular mass of about 550 kDa for the glycosylated form (Knott et al., 1986), apoB-100 is one of the largest monomeric proteins known (Chen et al., 1986). ApoB-100 is synthesized in the liver, from where it is secreted together with VLDL (Schaefer et al., 1978). ApoB-100 contains many hydrophobic regions some of which are extensive and associate tightly with lipids (Chen et al., 1989, Yang et al., 1989). The lipid binding structures in the form of amphipathic α-helices, β-strands, and hydrophobic regions span throughout the length of apoB-100 and consequently, unlike other apolipoproteins (C and E), apoB-100 binds irreversibly to the VLDL surface and does not exchange with other lipoprotein particles (Schaefer et al., 1978). Thus, apoB-100 remains bound to the shrinking particle throughout the metabolic cascade of transforming VLDL to LDL (Schaefer et al., 1978).

Each LDL particle contains a single copy of apoB-100, which has been suggested to be responsible for particle integrity by wrapping around the surface of LDL acting as a macromolecular scaffold (Chatterton et al., 1991, Chatterton et al., 1995, Liu&Atkinson 2011). However, rather than encircling an LDL particle in a straightforward manner, apoB-100 forms kinks and can be described as having a "ribbon and bow" configuration (Chatterton et al., 1995). According to this model the N-terminal 89% of the peptide wraps around the LDL as a thick "ribbon", completing the encirclement by about amino acid residue 4050, and the C-terminal 11% of the peptide constitutes the "bow" crossing the ribbon (Chatterton et al., 1995).

In addition to its suggested structural role, apoB-100 has a physiologically important function serving as a ligand for the LDL receptor (LDLR). Binding of apoB-100 to LDLR on the target cells initiates receptor-mediated uptake of LDL, the underlying mechanism of LDL clearance from circulation by hepatocytes (Brown et al., 1981). The relevance of this catabolic pathway is best illustrated by the genetic disorder familial hypercholesterolemia (FH), characterized by accumulation of high levels of LDL in the circulation due to mutations in the LDLR gene that hinder receptor-mediated clearance of plasma LDL (Defesche et al., 2017). The physiological upper limit for plasma LDL cholesterol (LDL-C) concentration has been suggested to be 1.5 mmol/l (Goldstein&Brown 1977), whereas in patients with the heterozygous form of FH plasma LDL-C level ranges from about 5 to 10 mmol/l and in the more severe homozygous form of FH, it may be as high as 30 mmol/l (Nordestgaard et al., 2013). Consequently, patients with FH have increased risk of premature development of atherosclerotic plaques.

The confirmation for the principal LDLR-binding site in apoB-100 came from studies of Borén and coworkers using site-directed mutagenesis of apoB-100 (Boren et al., 1998a) to indicate that the LDLR-binding site is formed by a cluster of basic amino acids spanning the residues 3359–3369 (termed as Site B). Accordingly, when the basic amino acids in the Site B were changed to neutral amino acids, LDL particles containing the uncharged Site B had defective receptor-binding indicating that Site B is functionally important for LDLR binding (Boren et al., 1998a). The group also showed that the highly conserved receptor-binding site is stabilized by the interaction of Arg3500 with Trp4369, and that a single point amino acid mutation, namely Arg3500 to Gln3500, completely abolishes interaction of LDL with the receptor (Boren et al., 1998a). This was shown to result from blocking of the LDLR-binding site by the C-terminus of apoB-100, which is kept away from the binding site by Arg3500 in wild-type LDL (Boren et al., 1998a). Because apoB-100 binds to the LDLR only after the conversion of VLDL to LDL, it is proposed that, in VLDL, the C-terminus normally functions to inhibit the interaction of apoB-100 with the LDLR, but after the conversion of VLDL to LDL, Arg3500 interacts with the C-terminus, permitting normal interaction between LDL and its receptor (Boren et al., 1998a). The Arg3500 to Gln3500 mutation in apoB-100, and to a lesser extent Arg3500 to Trp3500, is known to lead to a disorder known as familial defective apoB, where defective binding of apoB-100 to LDLR impairs LDL clearance from the circulation causing hypercholesterolemia in a similar manner to FH (Andersen et al., 2016).

ApoB-100 also mediates interaction of LDL with the arterial wall by binding to arterial PGs (Camejo et al., 1998). Interestingly, the principal PG-binding site appears to be formed by the same amino acid residues that form the LDLR-binding site (Boren et al., 1998b). The PG-binding ability of LDL has gained a vast interest in the context of atherosclerosis (Tabas et al., 2007, Boren&Williams 2016), as discussed in later sections.

## 1.3 High-density lipoprotein

HDL particles are the smallest and the densest lipoprotein particles of human plasma. The physiological role of HDL is opposite to that of LDL, thus, HDL removes cholesterol from the peripheral tissues and delivers it to liver for excretion into feces, a process referred to as reverse cholesterol transport (RCT) (Glomset 1968). Human plasma HDL constitutes a heterogeneous group of particles ranging 7–12 nm in diameter and 1.063–1.21 g/ml in density (Figure 3). By ultracentrifugation, these particles can be separated into two subfractions on the basis of their buoyant densities: HDL2 (1.063-1.125 g/ml) and HDL3 (1.125-1.21 g/ml) (Havel et al., 1955). These subspecies can be further divided in decreasing order of a particle diameter into HDL2b, HDL2a, HDL3a, HDL3b, and HDL3c with average diameters of 10.6, 9.2, 8.4, 8.0, and 7.6 nm, respectively (Blanche

et al., 1981). The calculated lipid composition of an HDL2 particle is on average 137 molecules of phospholipids (PL), 50 molecules of unesterified cholesterol (UC), 90 molecules of CE, and 19 molecules of TAG, whereas each HDL3 particle contains on average 51 molecules of PL, 13 molecules of UC, 32 molecules of CE, and 9 molecules of TAG (Shen et al., 1977).

On the basis of electrophoretic mobility human plasma HDL particles can be separated into two main subpopulations: spherical α-HDL particles, which have the same mobility as α-globulin and discoidal pre-β HDL particles, which are the precursors for the spherical α-HDL particles and migrate similarly to pre-β globulin (Kunitake et al., 1985, Castro&Fielding 1988). Two-dimensional electrophoresis allows further separation by charge and size of discoidal very small pre-β1, large pre-β2 and -β3, small discoidal α4, small spherical α3, medium-sized spherical α2, and large spherical α1 (Asztalos et al., 2005, Camont et al., 2011, Rosenson et al., 2011).

HDL can also be separated on the basis of apolipoprotein composition into several subpopulations using immunoaffinity methods (Hennessy et al., 1993). ApoA-I is the principal apolipoprotein of HDL particles comprising approximately 60–70% of total plasma HDL protein, with the second most abundant apolipoprotein, apoA-II, comprising about 20% (Huang et al., 2011). In human plasma, about 25% of apoA-I is present in HDL particles containing only apoA-I (LpA-I); the remaining HDL particles contain both apoA-I and apoA-II (LpA-I+A-II), typically in a molar ratio of 1-2/1 (Gauthamadasa et al., 2010). Moreover, a minor subpopulation of large, spherical HDL particles migrating to γ-position contain apoE as the only apolipoprotein (Huang et al., 1994). Other apolipoproteins that have been associated with HDL particles include apoA-IV, and –V, apoC-I, -II, -III, and –IV, apoD, apoF , apoH, apoJ, apoL-I, and apoM [summarized in (Rosenson et al., 2011)].

Altogether, over 200 lipid species and over 80 different proteins, including the HDL remodeling proteins cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT), the HDL oxidizing protein myeloperoxidase, the antioxidant protein paraoxonase1, and protease inhibitors (antithrombin III, α-1-antitrypsin inhibitor, serine protease inhibitors) have been proposed to be associated with HDL (Toth et al., 2013). Thus, hundreds of different HDL subspecies based on their individual protein and lipid composition is likely to be present in human plasma. However rather than representing static pools of distinct particles, they reflect the dynamic nature of HDL resulting from continuous remodeling, lipolysis, and fusion that can convert smaller particles to larger particles and vice versa, as will be discussed in the section 1.3.2.

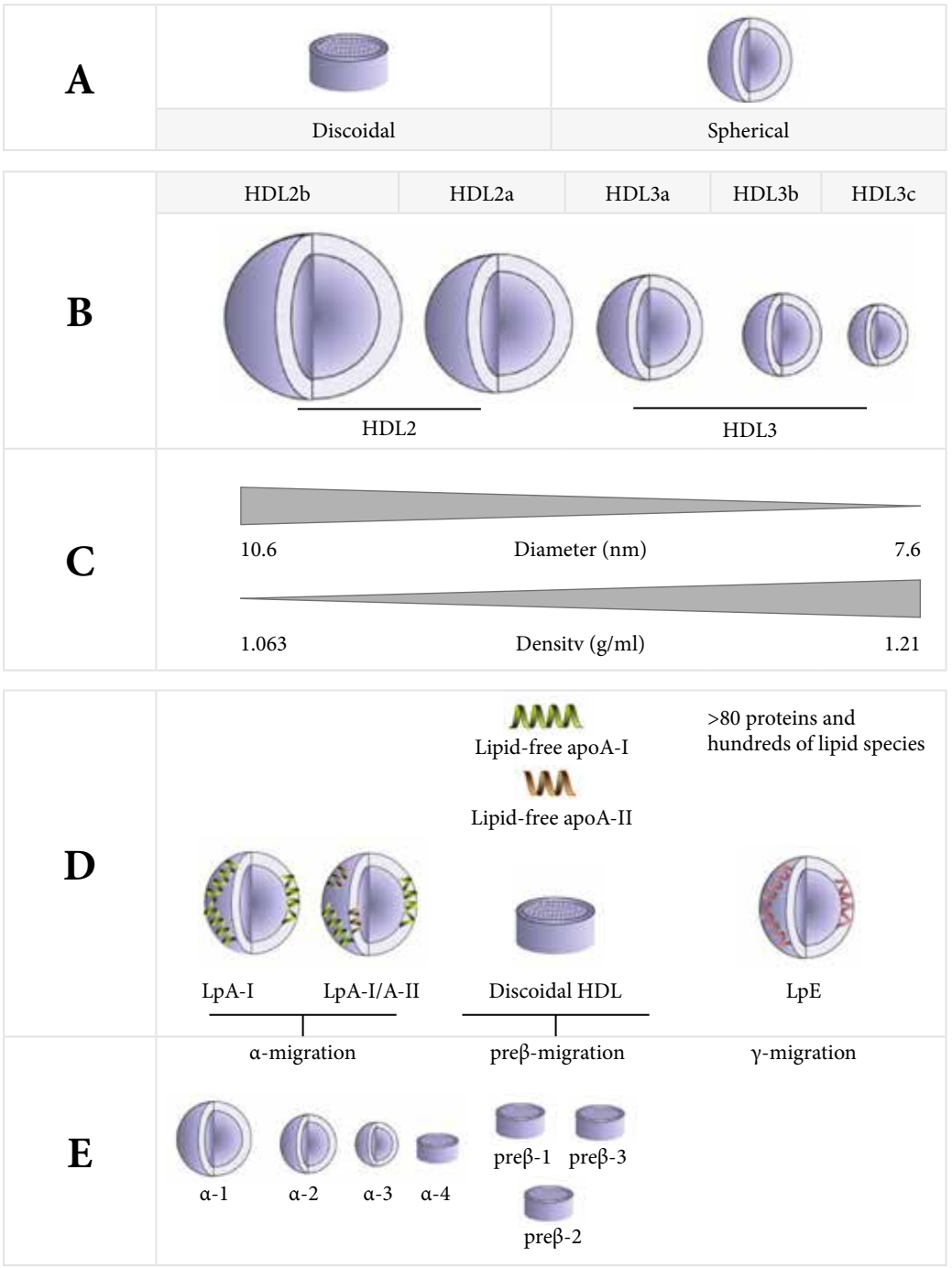



***Figure 3. HDL heterogeneity and complexity.*** *HDL particles in human plasma consist of various subclasses that can be distinguished based on shape (A), size (B), density (C), apolipoprotein and lipid composition (D), and electrophoretic mobility (E). Modified from (Rye et al., 2009) with the permission of the copyright holder.*

### 1.3.1 Apolipoprotein A-I

ApoA-I is the principal protein component of HDL particles. Human apoA-I is a 28 kDa protein consisting of 243 amino acids (Brewer et al., 1978, Breslow et al., 1982) and is made up of repeating amphipathic α-helices that reversibly bind it to the surface of HDL particles (Segrest et al., 1992). Indeed, in contrast to apoB-100, apoA-I is an exchangeable apolipoprotein capable of transitioning between lipid-bound and lipid-free states. The majority of circulating apoA-I is bound to HDL particles, whereas approximately 5–10% of apoA-I in the serum or extracellular space has been estimated to be relatively lipid free (Neary&Gowland 1987). The apoA gene is expressed in the liver and intestine (Zannis et al., 1982, Eisenberg 1984) and locates on chromosome 11 as a is part of the APOA1/C3/A4/A5 multigene cluster (Karathanasis 1985). The protein contains two key domains, an N-terminal α-helix bundle that spans residues 1 to 189 and a separately folded C-terminal domain spanning the remainder of the protein (Saito et al., 2003). Of these, the C-terminal domain is more hydrophobic and has higher lipid affinity compared with the N-terminus (Saito et al., 2004). ApoA-I plays crucial roles in HDL metabolism and RCT through its interaction with ATP-binding casette transporter A1 (ABCA1), activating LCAT, and acting as a ligand for hepatic scavenger receptor class B type 1 (SR-B1) (Mei&Atkinson 2015).

### 1.3.2 HDL metabolism and reverse cholesterol transport

The first step in the formation of HDL is the biogenesis of nascent discoidal HDL through efflux of cellular lipids to extracellular lipid-free or lipid-poor apoAI in a process mediated by ABCA1 (Wang&Smith 2014). The esterification of cholesterol through the action of the enzyme LCAT then leads to building up the hydrophobic core transforming the discoidal particles into spherical particles that represent the bulk of circulating HDL (Francone et al., 1989). The growing particles can accept additional cellular cholesterol through the activities of cellular ABCG1 (Wang et al., 2004) and SR-BI (Ji et al., 1997). Finally, the CEs in HDL are returned to the liver directly via hepatic uptake by SR-BI, or indirectly via transfer by CETP of CEs to VLDL and LDL, which then deliver CEs to liver. In the liver, the CEs can be converted to UC for direct excretion or converted to bile acids for excretion, completing the RCT pathway.

HDL particles are continuously remodeled in plasma by various proteins that determine the concentration, composition, and size of plasma HDL particles (Table 1). In addition to ABCG1, SR-B1, CETP these include phospholipid transfer protein (PLTP) that exchange lipids between HDL and other lipoproteins, and various lipases (lipoprotein lipase, endothelial lipase, and hepatic lipase) that hydrolyze HDL lipids. During the remodeling of HDL in plasma lipid-free apoA-I is continuously being regenerated, thus the assembly of HDL in the circulation may have a significant contribution to the total plasma HDL pool.

| *Table 1. Proteins involved in HDL metabolism and remodeling* | | |
|---|---|---|
| Protein | Function | HDL conversion |
| Cholesterol transporters | | |
| ABCA1 | Transfers PL and UC to apoA-I | apoA-I > discoidal |
| ABCG1 | Transfers UC to HDL | Spherical > larger spherical |
| SR-B1 | Transfers UC to HDL | Spherical > larger spherical |
| Enzymes | | |
| Endothelial lipase | Hydrolyzes PLs (and to a lesser extent TAGs) | Spherical > small spherical |
| Hepatic lipase | Hydrolyzes PLs and TAGs | Spherical > small spherical + free apoA-I |
| LCAT | Generates CEs | Discoidal > spherical |
| Lipid transfer proteins | | |
| CETP | Transfers CEs and TAGs between HDL, LDL, and VLDL | Spherical > small spherical + free apoA-I |
| PLTP | Transfers PLs between HDL a nd VLDL and between individual HDL particles | Spherical > large spherical and small spherical + free apoA-I |

*Data from (Ji et al., 1997, Wang et al., 2004, Rye&Barter 2014, Wang&Smith 2014)*

## 1.4 Pathophysiology of atherosclerosis

After analysis of the data from the Framingham study (Dawber et al., 1957), LDL-C, TG and HDL-C emerged as strong independent predictors of atherosclerosis, and remain to date the cornerstone in risk estimation for future atherosclerotic events. Increased plasma LDL-C and reduced HDL-C levels drive atherogenesis by promoting cholesterol accumulation within the arterial wall (Stamler et al., 1986). It has been reported that lipoproteins up to approximately 70 nm in diameter can cross an intact endothelium (Nordestgaard&Zilversmit 1988), and among these the smaller ones pass more readily than the larger ones. Thus circulating LDL (~ 20 nm) and HDL (~ 10 nm) can efficiently penetrate the endothelial cell (EC) lining of the artery wall (Stender&Zilversmit 1981, Nordestgaard et al., 1992) and thus to enter the innermost layer of the arterial wall, the intima. The smaller HDL particles also efficiently exit the intimal layer, whereas the larger LDL particles are more easily trapped within the intima, a phenomenon which promotes their intramural accumulation, and initiates series of events leading to atherosclerotic lesion formation (Williams&Tabas 1995, Tabas et al., 2007). The earliest recognizable gross lesions in the pathogenesis of atherosclerosis are called the fatty streaks (Stary et al., 1994), characterized by the appearance within the intima of cells, mainly macrophages

but also smooth muscle cells (SMCs), with high amounts of large intracellular CE-rich lipid droplets (Haley et al., 1977, Gerrity et al., 1979, Gerrity&Naito 1980, Schaffner et al., 1980). Such lipid-laden cells appear foamy in the electron microscope and are thereby referred to as foam cells (Takebayashi et al., 1972). As the atherogenic process proceeds, the continuous lipid accumulation and a complex series of cellular events gradually lead to development of more advanced lesions, which may ultimately predispose to clinical complications (Stary et al., 1994, Stary et al., 1995).

### 1.4.1 Humoral and parietal factors promoting atherogenesis

Although the central role of LDL in atherogenesis cannot be overstated, several other humoral and parietal factors are required to initiate and maintain the atherogenic process within arterial intima. Intima comprises the luminal endothelium and the underlying connective tissue and is composed of two layers: 1) a subendothelial PG-rich layer containing SMCs mainly of the synthesizing phenotype as well as isolated tissue macrophages near the endothelium, and 2) a deeper musculoelastic layer rich in collagen, elastic fibers, and layers of SMCs of the contractile phenotype (Stary et al., 1992). The intima is separated by an internal elastic lamina from the media, the middle layer of highly ordered structure of contractile SMCs layered within elastic fibers and collagen. Finally, the outermost layer surrounding the media, called the adventitia, is composed of loose connective tissue and hosts lymphatic veins and small blood vessels, vasa vasorum, through which nutrients and metabolic waste flux to and from the arterial wall (Nakano et al., 2005).

Within the intima LDL metabolism differs from that of other tissues. This difference is due to specific structural characteristics of intima, mainly the lack of capillaries and lymphatics within intima (Hulten&Levin 2009). As a consequence, the level of LDL-C within intima is 10-fold higher compared with other tissues (Smith 1990), which makes intima especially susceptible for development of atherosclerotic plaques.

Studies of the early events of atherogenesis have shown that lipoprotein accumulation in the arterial intima occurs preferentially at sites known to be susceptible for later plaque development (Schwenke&Carew 1989a, b, Nakashima et al., 2007). Such prelesional but lesion-prone sites are located preferentially at branch points and curvatures of the arterial tree (Stary et al., 1992). The altered hemodynamic parameters, such as low shear stress and disturbed flow at these sites are intrinsically linked to vascular endothelial phenotype and inflammation via activation of ECs and upregulation of adhesion molecules on their surface (Gimbrone&Garcia-Cardena 2013, Tabas et al., 2015). These adhesion molecules trigger the migration of circulating inflammatory cells to the subendothelial space (Rao et al., 2007). The same hemodynamic factors lead to changes in endothelial function in a

manner that impairs atheroprotective functions. Furthermore, physiological adaptation of the arterial wall to the altered hemodynamic forces at the lesion prone sites cause intimal thickening (Stary et al., 1992, Tabas et al., 2015). Thickened intima increases the transit distance of LDL to adventitial lymphatics or back to the vessel lumen (Kovanen 1990). Furthermore, proliferation of extracellular matrix (ECM) molecules, especially PGs that are capable of binding to and trapping LDL within the arterial wall, likely renders these sites especially prone to lesion formation (Stary et al., 1992, Nakashima et al., 2007, Steffensen et al., 2015).

### 1.4.2 Subendothelial retention of LDL within the arterial wall

Several lines of evidence support the concept that the underlying mechanism of cholesterol accumulation and thus initiation of atherogenesis is the focal retention of LDL by subendothelial PGs within the arterial intima (Boren&Williams 2016). The concept was extensively studied by Camejo and co-workers [reviewed in (Camejo et al., 1980a)] and reinforced later by Williams and Tabas in their 'response to retention' hypothesis of atherosclerosis (Williams&Tabas 1995, Tabas et al., 2007, Boren&Williams 2016), which was promoted by numerous *in vitro* and *in vivo* studies both in human and experimental animals. Such studies have shown that apoB-100-containing lipoproteins from plasma can interact *in vitro* with PGs extracted from the aortic wall (Bihari-Varga&Vegh 1967, Iverius 1972, Camejo et al., 1975, Camejo et al., 1980b, Vijayagopal et al., 1981) and that complexes of lipoproteins and PGs are also present *in vivo* in human atherosclerotic lesions (Srinivasan et al., 1972, Srinivasan et al., 1975, Camejo et al., 1985, Nakashima et al., 1985). Furthermore early LDL deposits are found in the arterial intima of experimental animals and precede further plaque development (Mora et al., 1987, Frank&Fogelman 1989, Nievelstein et al., 1991). Within the intima such accumulated lipoproteins are present enmeshed in the dense ECM (Frank&Fogelman 1989). Consistent with the studies in experimental animals Nakashima and coworkers (Nakashima et al., 2007) have indicated that human atherosclerosis begins with extracellular deposition of apoB-containing lipoproteins in the outer layer of the pre-existing adaptive intimal thickening rich in PGs.

Recent support for the importance of lipoprotein retention in atherogenesis has come from experimental studies in hypercholesterolemic animals [reviewed in (Boren&Williams 2016)]. In such studies interventions were performed that did not change plasma concentration of apoB-containing lipoproteins but specifically decreased or increased the retention of these particles within the arterial wall. Consequently, corresponding decrease or increase in atherogenesis was observed.

#### 1.4.2.1 Molecular mechanisms of LDL retention

The interaction of PGs and LDL is ionic in nature and occurs between the negatively charged GAG chains and the positively charged amino acid residues, such as Lys and Arg, of apoB (Camejo et al., 1998, Chait&Wight 2000). To date, at least eight potential binding sites for PGs have been identified *in vitro*, most of them close to the C-terminal end of the protein (Camejo et al., 1998). However, it is suggested that most of them may not be functionally available on LDL. Supporting this hypothesis, Borén and coworkers (Boren et al., 1998a) have shown that the binding of LDL particles to PGs is severely impaired by mutation of Lys3363 to Glu3363 (Boren et al., 1998a), thus implying that the apoB-100 in LDL contains only a single crucial PG-binding site, that is the amino acid residues 3359–3369 (referred to as Site B). Experimental studies have provided evidence that specific arterial wall PGs, particularly those containing chondroitin sulfate (CS) side chains have an important role in apoB-containing lipoprotein retention and atherosclerosis (Williams 2001, Gustafsson&Boren 2004). Furthermore, purified arterial CSPGs, particularly from lesion-prone sites, have high affinity for LDL (Cardoso&Mourao 1994, Ismail et al., 1994). The major CSPGs in the ECM of the mammalian arterial wall are versican, decorin and biglycan, and of these, apoB shows the strongest colocalization with biglycan (O'Brien et al., 1998).

While LDL retention within the arterial wall is initially related to direct binding between LDL and subendothelial PGs (Boren et al., 1998a, Skålen et al., 2002), indirect binding via accessory molecules acting as a 'bridge' between LDL and arterial matrix appears to govern as lesions progress (Gustafsson et al., 2007). Indeed, a series of studies in mice expressing apoB-100 with site directed mutations in its GAG-binding domain (Boren et al., 1998a, Skålen et al., 2002) demonstrated that the mutation in apoB-100 caused weak binding of the mutated apoB-containing LDL to PGs. The results showed convincingly that mice expressing the PG-binding–defective LDL had greatly reduced atherogenesis and that this effect was indeed due to decreased interaction of the mutated LDL with arterial wall PGs (Skålen et al., 2002). Further studies in such mice expressing the PG-binding defective LDL showed that although the initiation of atherosclerosis was delayed in these mice, they eventually developed the same level of atherosclerosis as mice expressing control LDL (Gustafsson et al., 2007). This was due to infiltration of the intima by macrophages that secrete bridging molecules, such as lipoprotein lipase that facilitate LDL adherence to arterial matrix. Such bridging molecules work together in sync with other possible proatherogenic mechanisms such as alterations in PG synthesis, including that mediated by lesional macrophages (Chang et al., 1998, Maor et al., 2000), presence of lesion-specific LDL-binding proteins (Lees et al., 2005), pro-retentive modifications of LDL (Auerbach et al., 1996, Pentikäinen et al., 2000, Plihtari et al., 2010), and decrease in intimal pH (Sneck et al., 2005, Lähdesmäki et al., 2012) culminating in enhanced retention of atherogenic lipoproteins. Indeed, once early lesions appear, lipoprotein

retention is accelerated (Schwenke&St Clair 1992), which is likely to further promote lipid accumulation and lesion development.

### 1.4.3 Modifications of LDL hinder their exit from the arterial wall

Characterization of extracellular lipid particles isolated from atherosclerotic intima have provided evidence that the retained LDL particles within arterial intima are subjected to various enzymatic and nonenzymatic modifications such as proteolysis, lipolysis, and oxidation (Öörni et al., 2000). *In vitro* such modifications have shown to promote aggregation and fusion of LDL particles, as described in the next sections, generating enlarged lipid particles with increased PG-binding properties. Morphologically and chemically such *in vitro* generated modified LDL particles resemble those found extracellularly within the atherosclerotic intima (Smith et al., 1967, Smith et al., 1968, Hoff&Bond 1982, Ylä-Herttuala et al., 1990, Öörni et al., 2000, Lehti et al., 2018). It has become obvious that modification of LDL particles is an important step in atherogenesis promoting both extracellular and intracellular lipid accumulation within the arterial wall (Öörni et al., 2000, Tabas et al., 2007, Lu&Gursky 2013). Moreover, the retained and modified LDL particles trigger various proinflammatory responses in the vessel wall which further promote lesion formation (Pentikäinen et al., 2000, Boren&Williams 2016).

#### 1.4.3.1 Oxidation

Oxidized LDL (oxLDL) is present in plasma and in atherosclerotic lesions, both in experimental animals and in humans (Haberland et al., 1988, Ylä-Herttuala et al., 1989, Steinberg 2009). Oxidation of LDL is a complex process during which both the protein and lipid components may undergo oxidative changes at various degrees. Various cell types present in atherosclerotic lesions, such as ECs, SMCs, and monocytes produce free radicals that are able to oxidize LDL (Heinecke et al., 1984, Morel et al., 1984, Cathcart et al., 1985). LDL oxidation by cultured cells is promoted by the presence of transition metal ions such as $Cu^{+}$ and $Fe^{2+}$ in the culture medium, however, metal ions can oxidize LDL even in the absence of cells (Heinecke et al., 1984, Steinbrecher et al., 1984). Whether metal ions play a role in LDL oxidation *in vivo* is, however, still a matter of debate (Yoshida&Kisugi 2010). The heme released from red blood cells is capable of oxidizing LDL *in vitro* and could be a potential oxidative agent also *in vivo* (Grinshtein et al., 2003). LDL oxidation can also be mediated via oxidizing enzymes such as lipoxygenase and myeloperoxidase (Yoshida&Kisugi 2010).

Due to the wide-ranging and complicated mechanisms of oxidation, oxLDL exists in multiple forms. Depending on the method and extent of oxidation as well as the presence

of other agents such as metal ions, oxLDL may contain various amounts of oxidative fatty acid derivatives both in the ester and free forms as well as their decomposition products, oxidized products of cholesterol, protein with oxidized amino acids and cross-links, and polypeptides with varying extents of covalent modification with lipid oxidation products. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are natural bi-products of lipid peroxidation that can oxidize LDL. The corresponding modified LDL particles, MDA- and 4-HNE –LDL are known to occur *in vivo* (Lampka et al., 2006, Takamura et al., 2017). The multiple forms of oxLDL exhibit a wide array of proatherogenic properties. These include induction of foam cell formation, cytotoxicity, chemotactic activity for monocytes, ability to increase expression of VCAM-1, and ability to induce expression and secretion of a wide variety of proinflammatory cytokines from macrophages (Steinberg 2009).

#### 1.4.3.2 Lipolysis and proteolysis

Within atherosclerotic arterial intima, two types of phospholipolytic enzymes, the secretory phospholipase A2 enzymes ($sPLA_2$) and the secretory sphingomyelinase (sSMase), capable of hydrolyzing the surface PLs of LDL particles have been detected (Öörni et al., 2000, Hanasaki et al., 2002, Wooton-Kee et al., 2004). Of these, sPLA2 hydrolyzes PLs to produce free fatty acids and a lysophospholids, whereas sSMase hydrolyzes sphingomyelin molecules into phosphocholines and ceramides. Indeed, lesional LDL shows signs of hydrolysis of both sphingomyelin and phosphatidylcholine (Öörni et al., 2000). *In vitro* studies have shown that both types of phospholipases may induce aggregation and fusion of LDL particles leading to increased PG binding of such particles (Hakala et al., 1999, Öörni et al., 2000, Hakala et al., 2001). If lipolysis of LDL particles is preceded by proteolysis, the PG-binding of LDL particles seems to be further increased (Plihtari et al., 2010). Hydrolysis of LDL by $sPLA_2$ has also been shown to result in enhanced lipid accumulation in macrophages thus promoting foam cell formation (Hanasaki et al., 2002, Wooton-Kee et al., 2004, Lähdesmäki et al., 2012).

In addition to phosholipolytic enzymes, other enzymes capable of hydrolyzing the lipid components of LDL particles within arterial intima include the lysosomal acid lipase (LAL) (Hakala et al., 2003) and cholesterylester hydrolase (CEH) (Torzewski et al., 1998). LAL is capable of hydrolyzing the CEs and TAGs of LDL and thus to generate fused LDL particles that are avidly taken up my macrophages (Hakala et al., 2003). CEH is an enzyme that hydrolyzes fatty acid esters of cholesterol and other sterols. There is evidence for modification of LDL by CEH in atherosclerotic lesions (Torzewski et al., 1998) leading to UC-rich particles that are, similar to LAL-modified LDL, efficiently taken up by macrophages (Torzewski et al., 2004). Moreover, LDL particles hydrolyzed by LAL or CEH are able to induce proinflammatory response resulting in secretion of IL-8 from macrophages (Hakala et al., 2006) and ECs (Suriyaphol et al., 2002).

In addition to lipases, atherosclerotic lesions contain several proteases capable of hydrolyzing the apoB-100 of LDL *in vitro*. These include both plasma-derived and locally synthesized proteases. To the former group belong proteases plasmin, kallikrein, and thrombin (Piha et al., 1995) and to the latter group the matrix metalloproteinases (MMPs) and lysosomal proteases cathepsins D, F, K, and S secreted by intimal macrophages and smooth muscle cells of the lesions (Sukhova et al., 1998, Hakala et al., 2003, Öörni et al., 2004, Plihtari et al., 2010). Additional sources of proteolytic enzymes in the arterial intima are the MCs (Kaartinen et al., 1994b, a). The proteolytic actions of MCs on lipoproteins are discussed in the later sections.

Given that various agents capable of modifying the protein and lipid components of LDL are present in atherosclerotic lesions, it is not surprising that apoB-100–containing lipid droplets isolated from such lesions show signs of various types of modifications such as combined proteolysis and lipolysis (Kruth&Shekhonin 1994). Even a partial loss of apoB-100, an important macromolecule providing for the structural integrity for LDL particle is likely to loosen the lipid packing on LDL surface. Loosening of the surface of LDL due to proteolysis can promote hydrolysis of the LDL core lipids. Indeed, the ability of CEH to hydrolyze CEs in LDL is facilitated by proteolytic cleavage of apoB-100 (Bhakdi et al., 1995). Treatment of LDL with trypsin, plasmin, or MMP-2 and-9, together with CEH induces formation of LDL-derived lipid droplets (Chao et al., 1992, Bhakdi et al., 1995, Torzewski et al., 2004), showing that combined action of CEH and a protease that cannot alone induce LDL fusion such as plasmin (Piha et al., 1995), can trigger formation of LDL-derived lipid droplets *in vitro*.

In addition to oxidized, lipolyzed, and proteolyzed LDL, other forms of (modified) LDL, such as small dense, electronegative, and especially desialylated LDL have been implicated in atherogenesis as well (Dousset et al., 1992, Öörni et al., 2000, Sanchez-Quesada et al., 2012). The following section underscores the second part of the response-to-retention hypothesis of atherogenesis stating that the retained and modified lipoproteins trigger a series of events within the artery wall that ultimately leads to the formation of atherosclerotic lesions (Williams&Tabas 1995, Boren&Williams 2016).

### 1.4.4 Cellular responses to modified LDL

The accumulated LDL particles in the arterial intima can be cleared by macrophages, phagocytic immune cells that have been specialized in scavenging debris from their surroundings. Macrophages are responsible for uptake of accumulated LDL to prevent cytotoxicity, tissue injury, inflammation, and metabolic disturbances. It is a well-known fact that modifications increase the uptake of LDL by macrophages, a phenomenon originally described by Drs Brown and Goldstein (Brown et al., 1979, Goldstein et al.,

1979). The increased uptake of modified LDL is owed to expression on macrophages of scavenger receptors (SRs), a group of pattern recognition receptors capable of recognizing and binding foreign or altered-self targets for further elimination (Canton et al., 2013). Modifications of LDL create recognition sites for SRs such as SR-A1 and CD36, which unlike the LDLR, are not subject to negative feedback regulation by intracellular cholesterol, allowing an unregulated accumulation of cholesterol within macrophages (McLaren et al., 2011, Chistiakov et al., 2017a). Over time, the macrophages become loaded with LDL-derived cholesterol and transform into foam cells, which are filled with cytoplasmic CE-containing lipid droplets (Chistiakov et al., 2017b). Although macrophages are the major cell type responsible for clearance of modified LDL in the arterial wall, the defense against the massive influx of modified LDL is partially fulfilled by a shift to a phagocytic phenotype of both the SMCs and endothelial cells (ECs) (Chaabane et al., 2014).

### 1.4.5 The stages of atherosclerotic development

The American Heart Association (AHA) has created a detailed classification scheme for atherosclerotic lesions that was designed to be used as a histological "template" or "reference" in the clinical setting (Stary et al., 1994, Stary et al., 1995). AHA recommends classification of atherosclerotic lesions into 6 different numerical categories (I-VI).

#### 1.4.5.1 Early lesions

The first isolated lipid-laden foam cells appear as a response to LDL accumulation typically in childhood and are considered the hallmark of the earliest stage of atherosclerotic lesion development, the type 1 lesion (Stary et al., 1994). Gradually, the accumulation of LDL sparks a local inflammatory response leading to activation of the resident arterial wall cells (Tabas et al., 2007). Modified LDL particles are able to activate the arterial ECs to express chemokines and cell adhesion molecules, such as monocyte chemotactic protein 1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and endothelial selectin (Cushing et al., 1990, Rajavashisth et al., 1990, Li et al., 1993, Leitinger et al., 1999, Zeuke et al., 2002). These molecules stimulate entry of inflammatory cells, mostly monocytes into the intima, where monocytes differentiate into macrophages and ultimately to foam cells (Rao et al., 2007, Hansson&Hermansson 2011). Owing to the lipid load, the foam cells lose the mobility and become unable to egress out of the arterial wall. Furthermore, modified LDL activates intracellular signaling networks in macrophages that induce production of various proinflammatory mediators such as chemokines, and cytokines (Stewart et al., 2010, Miller et al., 2012). Cumulatively, this has the effect of triggering a vicious cycle of recruiting more monocytes into the intima and of

opening up passages for the migration of SMCs from the media (Hansson&Hermansson 2011). Multiple layers of macrophage foam cells and lipid-laden intimal SMCs, as well as scattered droplets of extracellular lipids form a thin layer of yellowish streaks, the fatty streak or the type II lesions (Stary et al., 1994). Furthermore, the intimal SMCs switch from contractile to synthetic phenotype with enhanced production of ECM components. Such fatty streak lesions can usually be found in the aorta in the first decade of life, in the coronary arteries in the second decade, and in the cerebral arteries in the third or fourth decades (Lusis 2000). Intermediate or type III lesions appear in young adults and contain lipid-laden macrophages and vascular cells. Furthermore, continued lipid accumulation yields pools of extracellular lipids and particles that disrupt the coherence of some intimal SMCs (Stary et al., 1994). Common to such early lesions is that they are focal, relatively small, and clinically silent.

#### 1.4.5.2 Advanced lesions

As stated above, subclinical atheromatous changes within the arterial wall progress silently for decades. During these years more complex plaques develop through continuous LDL accumulation and a complex series of cellular processes. The initial response to the subendothelial retention of LDL as an appropriate attempt to clear unwanted and dangerous debris from the artery wall ultimately becomes maladaptive in advanced atherosclerosis largely due to altered behavior of arterial phagocytes which underlies defects in inflammation resolution (Hansson&Hermansson 2011). Advanced lesions are defined as lesions that are associated with intimal disorganization. The extracellular lipid that can already be found as pools in type III lesions is the immediate precursor of the larger, confluent, and more disruptive core of extracellular lipid that characterizes type IV lesions also known as atheroma (Stary et al., 1995). This dense lipid core is built from extracellular lipids, crystallized cholesterol, and cellular debris. Failure to suppress continuous immune cell recruitment, to clear apoptotic cells, and to induce immune cell egress exacerbates plaque inflammation. As monocyte-derived macrophages, T lymphocytes, MCs, dendritic cells, and neutrophils accumulate the lesions, the inflammatory status of the plaques is heightened (Hansson&Hermansson 2011). Atherosclerotic lesions most often undergo a partial resolution process characterized by the formation of a fibrous cap consisting of ECM, mostly collagen, produced by intimal SMCs on top of the lipid core (Libby 2008). Fibrotic responses characterize fibroatheromas or type Va lesions. When the lipid core and other parts of the lesion are calcified, type V lesion is referred to as type Vb, whereas a type V lesion that consists mainly of fibrous connective tissue and little or no accumulated lipid or calcium is referred to as type Vc. These lesions may narrow the arteries inducing end-organ ischemia, although formation of a thick lesion can occur without narrowing the arterial lumen, as well.

Over time certain types of atherosclerotic lesions develop features that can lead to acute clinical complications. These so-called vulnerable or high-risk plaques contain a large necrotic lipid core, thin fibrous cap, and a heightened inflammatory state. Such vulnerable plaques are prone to surface defects such as rupture and erosion of the fibrous cap (Mäyränpää et al., 2006) and ensuing development of luminal thrombosis that may lead to a stroke or sudden cardiac death. Plaques that develop rupture, hematoma, and/or thrombus are classified as type VI or complicated lesions (Stary et al., 1995).

## 1.5 Anti-atherogenic functions of apoA-I and HDL

As discussed thus far, increased plasma levels of LDL-C drive atherogenesis by promoting lipid accumulation within the arterial wall. On the contrary, plasma levels of HDL-C correlate inversely with risk of atherosclerotic cardiovascular disease (ACVD) (Castelli et al., 1986). However, the relationship between atherosclerosis and HDL is more complex than that between LDL and atherosclerosis. Apparently low HDL-C is associated with atherosclerotic disease not in a causal way but because low HDL-C is frequently associated with elevated TGs (Reiner 2013, Rader 2016). Indeed, a number of pharmacological and genetic studies aiming to develop HDL-raising therapies have failed to demonstrate that increased plasma levels of HDL-C would result in reduced ACVD risk (Toth et al., 2013). Such studies have given rise to a controversy over whether plasma levels of HDL-C as a static measure of cellular cholesterol carried by plasma HDL actually reflect functional quality of HDL (Parhofer 2015). Indeed, there is no convincing evidence to date that HDL-raising agents, including CETP inhibitors, small molecules that increase apoA-I synthesis, or niacin reduce clinical cardiovascular events (Toth et al., 2013). The interest in the therapeutic potential of HDL remains high due, to a large extent, to the strength of the circumstantial evidence provided by pre-clinical and (limited) clinical studies showing that HDL can promote the regression of atherosclerosis when the levels of functional particles are increased exogenously or endogenously (Feig et al., 2014, Rader 2016). Indeed, intravenous infusions of reconstituted HDL (rHDLs) particles consisting of complexes of PL and apoA-I as well as infusions of apoA-$I_{Milano}$, a mutant form of apoA-I discovered in Italian families with low HDL-C but apparently decreased CV risk, have been shown consistently in a variety of animal models to reduce experimentally induced atherosclerosis (Badimon et al., 1990, Rubin et al., 1991, Chiesa et al., 2002, Nicholls et al., 2005, Ibanez et al., 2008). Similar antiatherogenic effect of infusing rHDLs and apoA-$I_{Milano}$ into humans has been suggested by two proof-of-concept studies (Nissen et al., 2003, Tardif et al., 2007). The disconnect between the circumstantial evidence and the absence of positive results in human clinical outcome trials do not eliminate HDL from consideration as an atheroprotective agent, but emphasizes the important distinction between HDL functionality and the simple static plasma levels of HDL-C.

To support the cardioprotective functions of HDL, numerous observations from animal and basic studies have shown that HDL possesses several properties that are potentially antiatherogenic (Mineo&Shaul 2012). The best known of these effects is the ability of HDL to induce cholesterol efflux from macrophage foam cells and to promote RCT (Brown et al., 1980, Lee-Rueckert&Kovanen 2011). Multiple pathways are known by which excess cholesterol in foam cells can be removed by HDL (Ji et al., 1997, Bodzioch et al., 1999, Wang et al., 2004). However, transport of cellular lipids to lipid-free or lipid-poor apoA-I by ABCA1 is the key pathway regulating macrophage cholesterol homeostasis and thus playing a critically important role in atherosclerosis prevention and management. Indeed, patients with Tangier disease have not only very low HDL levels due to mutation of *ABCA1* (Bodzioch et al., 1999, Brooks-Wilson et al., 1999, Rust et al., 1999) but also suffer from excess cholesterol accumulation in tissues rich in macrophages, which express high levels of ABCA1 (Cavelier et al., 2001, Lawn et al., 2001, Oram&Lawn 2001). Subsequently, Tangier patients are at risk of premature atherosclerosis (Oram&Lawn 2001). Further support for the essential role of ABCA1-mediated cholesterol efflux pathway in the context of atherosclerosis has come from numerous studies in animal models showing that mice overexpressing apoA-I have increased, and mice deficient in apoA-I have reduced, macrophage RCT (Zhang et al., 2003, Moore et al., 2005), and that mice deficient in ABCA1 and mice in which ABCA1 has been selectively inactivated have increased atherosclerosis (Aiello et al., 2002, Westerterp et al., 2013).

Besides its classical role in regulating macrophage cholesterol homeostasis, HDL has plenty of other antiatherogenic properties. Indeed, HDL inhibits LDL oxidation and cytotoxic effects of LDL, promotes endothelial repair, improves endothelial function, has antithrombotic and anti-inflammatory properties, decreases white adipose tissue mass, increases energy expenditure, and inhibits the binding of monocytes to the endothelium (Cockerill et al., 1995, Morgantini et al., 2011, Mineo&Shaul 2012, Gordts et al., 2014). Many of the atheroprotective functions of HDL are attributed to apoA-I and are mediated by apoA-I interaction with ABCA1 (Mineo&Shaul 2013, Vuilleumier et al., 2013).

## 2 Mast cells

MCs are tissue-dwelling cells of hematopoietic origin currently emerging as multifunctional effector cells in numerous settings. MCs have become widely known for their detrimental actions in diseases such as allergic asthma, contact dermatitis, rheumatoid arthritis, atherosclerosis, and cancer (Eklund 2007, Balzar et al., 2011, Dudeck et al., 2011, Bot et al., 2015, Rao et al., 2015), whereas less well advertised is the beneficial action of MCs, most notably in the context of host defense toward insults by bacteria, parasites, and various toxic substances (Echtenacher et al., 1996, Malaviya et al., 1996, Maurer et al., 2004, Schneider et al., 2007, Akahoshi et al., 2011, Matsuguchi 2012, Cruz et al., 2014). The capability of MCs to participate in various processes, both of detrimental and beneficial nature, is attributed to a wide variety of inflammatory molecules such as histamine, heparin proteoglycan (PG), cytokines, chemokines, lipid-derived mediators, and various neutral proteases released by MCs upon activation. Many of these mediators, most notably histamine, heparin, and neutral proteases, are stored within abundant cytoplasmic secretory granules of MCs, specific intracellular organelles, whose characteristic staining property (Figure 4), termed metachromasia, initially led to the identification of these effector cells in the late 19$^{th}$ century (Wernersson&Pejler 2014).

Since the discovery of MCs, it has become obvious that MCs do not represent a homogeneous population of cells but display marked plasticity in their morphological, histochemical, biochemical, and functional characteristics depending on their anatomic localization (Metcalfe et al., 1997). In humans, the most precise marker of MC heterogeneity is the neutral granule protease content. Two major MC subtypes within human tissues distinguishable by their protease phenotype have been established (Irani et al., 1986, Irani et al., 1989), however, increasing evidence now suggests that the phenotypic heterogeneity of human MCs may actually be more complex (Weidner&Austen 1993, Andersson et al., 2009, Abonia et al., 2010, Dougherty et al., 2010, Andersson et al., 2011, Andersson et al., 2018).

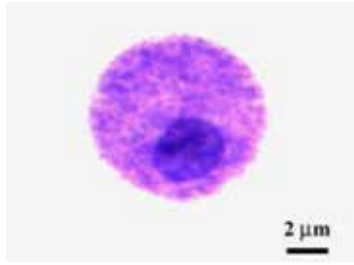



***Figure 4. Human MC displaying metachromatic staining.*** *Human MCs were cultured for 9 weeks and stained with toluidine blue, which turns the cytoplasmic secretory granules of MCs from a blue to a reddish color due to interaction of the basic dye with the highly sulfated glycosaminoglycans, notably heparin, within MC secretory granules. Image from (Lappalainen et al., 2007).*

## 2.1 Mast cell origin and differentiation

MCs derive from hematopoietic stem cells in the bone marrow. However, MCs are rather unique among cells of hematopoietic origin in that they undergo only part of their differentiation in the bone marrow, with the bulk differentiation occurring in peripheral tissues under the influence of the local microenvironment (Okayama&Kawakami 2006). The bone marrow origin of MCs was first demonstrated through a series of *in vivo* reconstitution studies using genetically MC-deficient mutant mice (Kitamura et al., 1978, Kitamura et al., 1981). In the early 1990s, the origin of human MCs from pluripotent $CD34^+$ progenitor cells in the bone marrow was demonstrated (Kirshenbaum et al., 1991). However, MCs do not complete their differentiation in the bone marrow, but leave the bone marrow as immature precursors and circulate in the blood as agranular leukocytes (Dahlin&Hallgren 2015). *In vitro* human MCs can be differentiated from peripheral blood-derived progenitors expressing a CD34 and a receptor tyrosine kinase for stem cell factor/kit ligand (SCF/KITLG) designated as KIT (Agis et al., 1993, Kirshenbaum et al., 1999). Recently a human tissue MC–like cells were developed *in vitro* from a population of peripheral blood-derived Lin(-) CD34(hi) CD117(int/hi) FcεRI(+) cells, suggesting that this rare population of precursor cells may represent circulating progenitors that give rise exclusively to MCs (Dahlin et al., 2016).

From the circulation, the MC progenitors are recruited into various peripheral tissues, where they complete their differentiation into mature MCs. From murine studies, it has become apparent that MC homing is a precisely controlled process, where integrin adhesion molecules play an important role (Hallgren&Gurish 2011, Dahlin&Hallgren 2015). Human MC progenitors have been reported to use α4β1-integrin for adhesive interactions with human vascular endothelium (Boyce et al., 2002). Furthermore, human MC progenitors express several chemokine receptors, CXCR2, CCR3, CXCR4, and CCR5, and respond *in vitro* to their ligands IL-8, eotaxin, stromal cell-derived factor (SDF)-1α, and macrophage inflammatory protein (MIP)-1α, respectively (Ochi et al., 1999).

The details of human MC differentiation within tissues have not been fully covered, however it is known that human MCs undergo optimal development in the presence of KITLG (Irani et al., 1992b, Valent et al., 1992, Mitsui et al., 1993). Stimulation of MCs by KITLG promotes differentiation, proliferation, maturation, and survival of MCs and their progenitors (Okayama&Kawakami 2006), while deprivation of KITLG results in rapid apoptosis of MCs both *in vivo* and *in vitro* (Iemura et al., 1994). KITLG elicits its functions through KIT, which is expressed on the surface of MCs throughout their development, unlike in other cells of hematopoietic origin that express KIT only during their early stages of development (Miettinen&Lasota 2005). The importance of KIT signaling in MC development is stressed by the 4 following facts: first, mice with

defects in KITLG (Sl/Sld mutants) or KIT (W/Wv mutants) are strikingly deficient in mature tissue MCs (Kitamura et al., 1978, Kitamura&Go 1979); second, in all variants of mastocytosis characterized by uncontrolled MC proliferation activating mutations of KIT are found (Bibi et al., 2014); and third, injection of KITLG into experimental animals (Galli et al., 1993) and human subjects results in MC hyperplasia (Costa et al., 1996). Consistent with this latter finding, subcutaneous administration of recombinant rat KITLG locally repaired the MC-deficiency in Sl/Sld mutant mice (Zsebo et al., 1990).

## 2.2 Mast cells are multifunctional effector cells

Mast cells are widely distributed throughout body and can be found in virtually all vascularized tissues especially at sites close to or in contact with the outside world, for example in the skin and the mucosal surfaces of the respiratory and gastrointestinal tracts (Galli et al., 2005). The localization of MCs at sites close to the external milieu reflects their role as being among the first cells of immune system to interact with environmental antigens and allergens, invading pathogens or environmental toxins (Metz&Maurer 2007). MCs are best known for their prominent role in IgE-associated hypersensitivity reactions and allergic disorders, however, it has become obvious that MCs can contribute to many processes of both innate and adaptive immunity and can exert both protective and pathogenic functions (Metz&Maurer 2007, Galli et al., 2008). Such effector functions include killing pathogens, degrading potentially toxic endogenous peptides or components of venoms, and regulating the numbers, viability, distribution, phenotype or non-immune functions of structural cells, such as fibroblasts and vascular endothelial cells (Metz et al., 2006, Dawicki&Marshall 2007, Kovanen 2007a, Galli et al., 2008).

MCs mediate their effector functions by releasing upon activation a wide range of preformed and de novo-synthesized mediators such as histamine, heparin, neutral proteases, arachidonic acid-derived lipid mediators, numerous cytokines, chemokines, and growth factors (Theoharides&Cochrane 2004, Grimbaldeston et al., 2006, Metz&Maurer 2007) (Table 2). MCs can also have positive, as well as negative immunomodulatory roles *in vivo*. These roles reflect the ability of MCs to influence recruitment, survival, development, phenotype or function of immune cells, including granulocytes, monocytes/macrophages, dendritic cells, T cells, B cells and natural killer cells (Galli et al., 2008). Through these effector and immunomodulatory functions, which can either promote or suppress certain features of an immune response, depending on the individual biological setting, MCs can contribute to numerous physiological and pathophysiological conditions such as host defense, innate and adaptive immune responses, homeostatic responses, angiogenesis, wound healing, tissue remodeling, and immunoregulation (Dawicki&Marshall 2007, Metz&Maurer 2007, Galli et al., 2008, Rao&Brown 2008, Maltby et al., 2009). Crucial

functions for MCs in a variety of disorders such as rheumatic arthritis (Eklund 2007), multiple sclerosis (Theoharides et al., 2007a), fibrosis (Holdsworth&Summers 2008), tumor growth (Maltby et al., 2009), inflammatory bowel disease (He 2004), and cardiovascular diseases, such as atherosclerosis (Kovanen 2007a), has been established ensuring that MCs are currently receiving increased attention among the scientific field outside of the allergy-related programs of research.

### 2.2.1 Mast cells release a wide variety of inflammatory mediators upon activation

MCs are equipped with an arsenal of cell surface receptors, through which they can be stimulated to release the wide array of biologically active compounds. The receptors include those for immunoglobulin (Ig) A (FcαR), IgE (FcεRI), and IgG (FcγRI, FcγRIIA, FcγRIIB, FcγRIII), receptors for C3α (C3α receptor) and C5α (C5α receptor), Mrgprb2/ receptor (a receptor for basic secretagogues) and its human ortholoque MRGPRX2 (McNeil et al., 2015), and toll like receptors (TLRs) 1-9 (Theoharides et al., 2012). The classical mechanism of MC activation is crosslinking via an IgE-specific antigen or allergen of IgE bound to its high affinity receptor, FcεRI, on the surface of MC (Galli&Tsai 2012). Crosslinking activates the FcεRI signaling pathway leading to acute release of preformed mediators, such as histamine, heparin, and neutral proteases stored within the cytoplasmic secretory granules, in an exocytotic process called degranulation. In addition to allergic triggers, MCs can be activated by numerous other immunological and non-immunological signals such as anaphylatoxins, antibody light chains, bacterial and viral antigens, various cytokines and chemokines, neuropeptides, chemical agents, and physical stimuli (Metz&Maurer 2007, Theoharides et al., 2007b). At later stages upon activation, MCs can also release newly synthesized mediators such as various cytokines, chemokines, arachidonic acid-derived lipid mediators, and growth factors. Depending on the mechanism of activation and the strength of the activating stimulus, MCs may release mediators belonging to either category (preformed mediators and newly synthesized mediators) or they may secrete distinct subsets of mediators through selective release without degranulation. Example of the former is IgE-mediated degranulation, whereas the latter may happe for example upon binding of lipopolysaccharide (LPS) to TLR-4 expressed on the MC surface (Theoharides et al., 2007b).

## 2.3 Mast cell neutral proteases

The major group of preformed mediators based on abundance is the neutral proteases, proteolytic enzymes having a neutral-to-slightly-basic pH optimum, which can account for up to 35% of the total cellular protein (Hellman and Thorpe, 2014). The neutral proteases are stored in high amounts as proteolytically active enzymes within the MC cytoplasmic granules (Pejler et al., 2007) embedded in a matrix of heparin PGs (Kovanen, 1993). Although stored in their fully active form, the acidic pH inside the granules restricts their activity during storage (Wernersson and Pejler, 2014). Once MCs are triggered to degranulate they expel some of their granules into the extracellular spaces, where proteases remain bound to heparin forming proteolytically active granule remnant s ready to act on their targets (Figure 5).

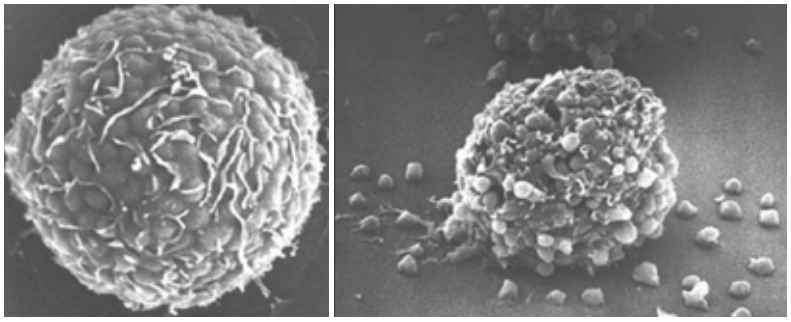


***Figure 5. Scanning electron micrographs of a resting and a degranulating rat serosal MC.*** *Under normal conditions, MCs are found in tissues in their resting state storing the various neutral proteases, histamine, and various preformed mediators in their cytoplasmic granules (Left panel). However, upon activation with an appropriate stimulus, such as crosslinking of receptor-bound IgE by an antigen, MCs acutely exocytose their preformed mediators in a process called degranulation (Right panel). In the extracellular fluid, histamine and other soluble mediators detach from heparin and diffuse away, whereas a fraction of the exocytosed neutral proteases remain bound to the granule heparin proteoglycan matrix, thereby forming a proteolytically active granule remnant (Kokkonen&Kovanen 1990).*

| *Table 2. Mast cell granule-associated mediators* | | |
|---|---|---|
| Biogenic amines | Enzymes | Proteoglycans |
| Dopamine | Arylsulfatases | Serglycin |
| Histamine | Carboxypeptidase A3 | Glycosaminoglycans |
| Serotonin | Cathepsin G | Heparin |
| Growth factors and Cytokines | Chymase | Chondroitin sulfate |
| IL-1, IL-3, IL-4, IL-5, IL-6, TNF-α, | β-Glucuronidase | Chemokines |
| IL-10, IL-13, GM-CSF, KITLG, TGF-β | Granzyme B | MIP-1α, MIP-1β |
| | Granzyme H | IL-8, RANTES, MCP-1, eotaxin |
| | Kinogenases | Lipid-derived mediators |
| | MMP-9 | Leukotriene B4 |
| | Phospholipases | Leukotriene C4 |
| | Peroxidases | Platelet activating factor |
| | Tryptase | Prostaglandin D2 |

*GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; MIP:, macrophage inflammatory protein, MMP-9, matrix metalloproteinase-9; RANTES, regulated upon activation normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α; KITLG, kit ligand; Data from (Shakoory et al., 2004, Rönnberg et al., 2014, Wernerssone&Pejler 2014).*

Neutral protease activity was first demonstrated in MCs (of human, dog, rat, mouse, rabbit) in the early 1950s by the use of histochemical technique involving cleavage of a chromogenic substrate [reviwed in (Nakamura et al., 2009)]. Further studies in the late 1950s characterized this activity as a chymotrypsin-like enzyme on the basis of its preference of hydrolyzing acetylethyl esters of aromatic amino acids, but of not basic amino acids (Benditt&Arase 1959). A year later, using other substrate-based techniques (Glenner&Cohen 1960), human skin MCs were reported to contain substantial levels of trypsin-like enzyme activity. However, it took over 20 years until tryptase and chymase responsible for trypsin-like and chymotrypsin-like enzyme activities, respectively, were first isolated and characterized (Schwartz et al., 1981, Schechter et al., 1983). Nowadays various neutral proteases, namely chymase, carboxypeptidase A3 (CPA3), cathepsin G, granzyme B, and tryptases of four isoforms have been identified within the granules of human MCs (Caughey 2016). Furthermore, a novel protease, granzyme H was reported in 2014 to be present within human MCs (Rönnberg et al., 2014). Apart from CPA3, which is a zinc-containing exopeptidase of the metalloproteinase family (Pejler et al., 2009), all MC neutral proteases are serine proteases characterized by an active site serine residue.

### 2.3.1 Tryptase

Tryptase is present in most, if not all, human MCs, and is the major neutral protease stored in human MC granules in terms of abundance (Metcalfe et al., 1997). Tryptase has trypsin-like cleavage specificity, as it cleaves protein substrates at the C-terminal side of arginine and lysine residues (Schwartz et al., 1981, Tanaka et al., 1983). However, unlike the many tryptic peptidases associated with digestion, hemostasis, clot lysis, and complement activation, tryptase is highly selective regarding its peptide and protein targets (Schwartz et al., 1981, Tanaka et al., 1983), which underscores its unique position in the hierarchy of peptidases belonging to the trypsin family. The human MC tryptase locus resides on chromosome 16p13.3 and spans approximately 1.6 Mb (Pallaoro et al., 1999). To date, four MC tryptase genes (TPSAB1, TPSB2, TPSD1, and TPSG1) plus various pseudogenes have been identified in humans (Pallaoro et al., 1999, Caughey et al., 2000). These fall into two major groups: the soluble α-, β-, and δ-tryptases and the membrane-anchored γ-tryptase, also known as transmembrane tryptase or TMT (Pallaoro et al., 1999). Of these, the β-tryptase appears to be the main form stored in human MC granules, and it occurs in three almost identical forms: βI, βII, and βIII (Miller et al., 1990, Vanderslice et al., 1990). MC tryptase has several unique features, one of the most remarkable ones being its organization into a tetrameric state with the active sites oriented toward a narrow central pore, and its consequent resistance to endogenous macromolecular protease inhibitors, such as serpins and α2-macroglobulin (Pereira et al., 1998).

Among the α-tryptases, two very similar forms have been identified in humans: αI and αII (Miller et al., 1989, Pallaoro et al., 1999). In contrast to β-tryptase, which is stored in the secretory granules and not released unless the MCs have been challenged by a degranulating stimulus, α-tryptase appears to be constitutively released via a selective pathway and is present at low levels in circulation even without MC degranulation (Schwartz et al., 1995). However, the activity of human α-tryptase is extremely low compared with β-tryptase, which is partly due to the amino acid substitution of glycine for asparagine at the position 216 of the substrate-binding pocket (Huang et al., 1999). In some individuals elevated basal serum tryptase levels are found due to increased copy number of α-tryptase (Lyons et al., 2016)

From δ-tryptase, two nearly identical forms (δI and δII) have been identified (Wang et al., 2002). However, the activity of δ-tryptase is also much lower than that of β-tryptase, which is mainly due to a premature stop codon that results in a truncated protein and affects the substrate specificity of δ-tryptase significantly (Wang et al., 2002).

Finally, two different forms of the human transmembrane tryptase (γ-tryptase) have been identified: γI and γII (Caughey et al., 2000). The γ-tryptases contain an extended

hydrophobic C-terminal domain followed by a small cytoplasmic tail, are anchored in either the plasma membrane or the secretory granule membrane, and only act locally upon MC activation (Caughey et al., 2000).

### 2.3.2 Chymase

In humans, the major chymotryptic protease, as defined by its preference for cleaving peptide and protein substrates at the C-terminal site of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan, is chymase (Powers et al., 1985, Caughey 2007). In contrast to rodents, which express several chymase isoforms (Pejler et al., 2007), only one chymase gene (CMA1) has been found in humans (Caughey et al., 1993). Chymase is specifically expressed in MCs (Caughey 2016). Human MC chymase is located on chromosome 14q11.2 (Caughey et al., 1993) at the end of a small cluster of four genes covering approximately 130 kb. This cluster also contains the cathepsin G gene (CTSG) and the granzyme H and B genes (GZMH and GZMB, respectively) (Caughey et al., 1993).

The human chymase is active in its monomeric form (Pejler et al., 2007) and has more destructive potential than tryptase, given that it can cleave a fairly wide variety of peptide and protein targets (Caughey 2007). Befitting its greater destructive potential, chymase is more susceptible to inhibition by circulating and extravascular anti-peptidases, including serpins and α2-macroglobulin, and is thus quickly inhibited after release, although some protection against inhibition is gained by tight binding to co-released PGs, notably the heparin PGs (Pejler&Berg 1995, Lindstedt et al., 2001, Caughey 2007). Chymase is closely associated with cardiovascular disorders (Kovanen 1997). It is activated in pressure-overloaded hearts and is able to convert angiotensin I to angiotensin II independently of the angiotensin-converting enzyme (Doggrell&Wanstall 2005).

### 2.3.3 Carboxypeptidase A3

Human CPA3 is a zinc-dependent metalloexoprotease that belongs to the carboxypeptidase (CP) A/B family (Arolas et al., 2007). As indicated by the letter "A" in its name, CPA3 has a CPA-like cleavage specificity, i.e., it prefers cleaving peptide and ester bonds at the amino side of the C-terminal aromatic amino acids (Goldstein et al., 1989). Human CPA3 is encoded by a single gene (CPA3), which situates on chromosome 3q24 (indicated by number 3 in its name) and spans over 32 kb (Pejler et al., 2007). It has been identified in the $MC_{TC}$ and in MCs containing tryptase and CPA3 but not chymase (Goldstein et al., 1989, Abonia et al., 2010, Dougherty et al., 2010). Despite having a CPA-like substrate-binding pocket and enzyme activity, CPA3 is structurally similar to bovine and human pancreatic CPB, which indicates its uniqueness among CPs (Goldstein et al., 1989, Reynolds et al., 1992).

### 2.3.4 Cathepsin G

Human cathepsin G is a serine protease that belongs to the cathepsin class of enzymes (Kryczka&Boncela 2017). Unlike tryptase, chymase, and CPA3, which are considered MC-specific proteases, expression of cathepsin G is traditionally related to neutrophils (Korkmaz et al., 2008). However, studies have provided evidence that also other cells, such as monocytes and macrophages, do express cathepsin G (Wang et al., 2014). Only one cathepsin G gene (*CTSG*) has been identified in humans, and it is located within a cluster of four genes on chromosome 14q11.2 (Caughey et al., 1993). Similar to chymase, cathepsin G has chymotrypsin-like activity, but, in terms of its destructive potential, cathepsin G is a weaker enzyme than chymase, and it also has a broader peptidase specificity; that is, it exhibits the unusual property of having both chymotryptic and tryptic activity (Caughey 2007).

### 2.3.5 Granzyme B

Granzymes are serine proteases classically known as granule components of cytotoxic T lymphocytes and natural killer cells (Lieberman 2003). After being released by these cells granzymes induce apoptosis in target cells, thus eliminating cells that have become cancerous or have been infected with bacteria and viruses (Bots&Medema 2006). Five granzyme subtypes have been identified in humans (A, B, H, K, and M) (Barry&Bleackley 2002), granzyme B being the most thoroughly characterized of them (Ngan et al., 2009). Granzyme B was first identified within human MCs in 2007 (Strik et al., 2007). It has been reported to be present in cultured human MCs and in MCs of human skin (Strik et al., 2007; Rönnberg et al., 2014). Granzyme B is a caspase-like serine protease that cleaves substrates at the carboxyl side of acidic residues, particularly aspartic acid (Fan&Zhang 2005). The granzyme B of lymphocytes and NK cells is a pro-apoptotic protease, which requires perforin to form pores in the plasma membrane of the target cells to help its entry into intracellular compartments (Fan&Zhang 2005). Unlike lymphocytes, MCs do not store perforin in their secretory granules (Pardo et al., 2007), which suggests a unique function of MC-derived granzyme B. Human MC-derived granzyme B is released from MC secretory granules upon activation (Strik et al., 2007). Extracellular granzyme B is able to cleave a number of ECM proteins (Hellman&Thorpe 2014), yet the understanding of the extracellular effects of human MC granyme B is limited. Recently it was, however, reported that by secreting matrix-degrading granzyme B MCs may alter the proliferative and organizational state of endothelial cells leading to reduced efficacy of anti-angiogenic therapy (Wroblewski et al., 2017).

### 2.3.6 Granzyme H

Another granzyme, namely granzyme H, which is constitutively expressed at high levels in human natural killer cells was recently identified in cultured human MCs and MCs of human skin (Rönnberg et al., 2014). As a member of a granzyme family, granzyme H is a death-inducing protease. On the contrary to granzyme B, granzyme H appears to be able to induce caspase-independent cell death (Fellows et al., 2007), although induction of caspase-dependent apoptosis of target cells by granzyme H has also been reported (Hou et al., 2008). In human MCs granzyme H and B seem to be reciprocally regulated (Rönnberg et al., 2014)supporting earlier findings obtained in T lymphocytes and natural killer cells (Sedelies et al., 2004)

## 2.4 Mast cell heterogeneity

Human MCs have traditionally been classified into two major subsets, the $MC_T$, which contains only tryptase and the $MC_{TC}$, which contains both tryptase and chymase (Irani et al., 1986) as well as carboxypeptidase A3 (CPA3) and cathepsin G (Schechter et al., 1990, Irani et al., 1991). This classification is based on the classical studies of Schwartz and his group (Irani et al., 1986, Irani et al., 1989) using newly developed antibodies against tryptase and chymase, that had, as mentioned, been isolated and characterized a couple of years earlier. Since the studies of Schwartz and coworkers, the concept of the existence of two MC subtypes, the $MC_T$ and the $MC_{TC}$ have held tight, although two 'additional' MC phenotypes namely $MC_C$ designating a MC containing chymase only (Weidner&Austen 1993), and a MC containing tryptase and CPA3 but not chymase have been identified within human tissues (Weidner&Austen 1993, Abonia et al., 2010, Dougherty et al., 2010). However, many researchers have failed to detect the $MC_C$ phenotype within human tissues (Irani et al., 1989, Tetlow&Woolley 1995, Gotis-Graham&McNeil 1997) providing a plausible explanation why its existence has often been neglected. An MC containing tryptase and CPA3, again, has been relatively recently (in 2010) identified (Abonia et al., 2010, Dougherty et al., 2010) but has not, at least yet, gained an established status as a major human MC phenotype.

| *Table 3. Distribution of $MC_T$, $MC_{TC}$, and $MC_C$ in normal human tissues* | | | |
|---|---|---|---|
| Tissue | % $MC_T$ | % $MC_{TC}$ | % $MC_C$ |
| Skin | <1 | >99 | |
| Lung | | | |
| Alveoli | 91/93 | 8/7 | 1/- |
| Bronchi | 78 | 10 | 12 |
| Bronchial epithelium | 100 | 0 | - |
| Bronchial subepithelium | 75 | 25 | - |
| Axillary lymph nodes | 1 | 97 | 2 |
| Breast parenchyma | 1 | 99 | 0 |
| Stomach | | | |
| Mucosa | 52 | 39 | 9 |
| Submucosa | 0 | 73 | 27 |
| Small intestine | | | |
| Mucosa | 65/81 | 31/19 | 4/- |
| Submucosa | 0/23 | 76/77 | 24/- |
| Colon | | | |
| Mucosa | 53 | 37 | 10 |
| Submucosa | 0 | 96 | 4 |
| Nasal mucosa | 66 | 34 | - |
| Conjunctiva | 5 | 95 | - |
| Synovium | 17/34 | 83/66 | -/- |
| Heart | 10 | 90 | - |
| Kidney | 65 | 35 | - |
| Uterus | | | |
| Endometrium | 84 | 16 | - |
| Inner myometrium | 48 | 52 | - |
| Outer myometrium | 10 | 90 | - |
| Cervix | 40 | 60 | - |

*Data from (Weidner&Austen 1993, Irani&Schwartz 1994, Sperr et al., 1994, Mori et al., 1997, Buckley et al., 1998, Gotis-Graham et al., 1998, Yamada et al., 2001). Data are expressed as mean. -, Data not available; /, different values given by the authors.*

### 2.4.1 Tissue distribution of human mast cell subtypes

Intially, the human $MC_T$ and $MC_{TC}$ subtypes were suggested to be the equivalents of the previously described 'mucosal' and 'connective' tissue MCs of rodents, respectively, but their tissue distribution is not as clearly demarcated as that in rodents. Different groups of investigators have reported quite different proportions of $MC_T$ and $MC_{TC}$ subtypes in various human tissues. However, some rules appear to govern the tissue distribution of $MC_T$ and $MC_{TC}$ subtypes. In histologically normal human tissues, $MC_T$ is the primary subtype at the mucosal surfaces of the respiratory and gastrointestinal tracts, such as in the lungs, particularly the alveoli, and the small intestinal mucosa, whereas $MC_{TC}$ is the predominant subtype found in the skin, synovium, and gastrointestinal submucosa (Table 3). Although a particular MC subtype seems to predominate in a particular tissue, a fraction of the other subtype is also usually present. Indeed, most human tissues contain a mixed population of $MC_T$ and $MC_{TC}$ subtypes, their relative abundances changing with inflammation and other disease processes (Table 4).

*Table 4. Relative abundances of $MC_T$ and $MC_{TC}$ in normal and diseased human tissues*

| MC subtype | Synovium | | | Kidney | | | Carotid Artery | | |
|---|---|---|---|---|---|---|---|---|---|
| | Normal | Early RA | Late RA | Normal | Rejected nephrect. specimens | Rejected biopsy specimens | Normal | Early lesion | Advanced lesion |
| $MC_T$ | 17% | 72% | 37% | 65% | 65% | 71% | 10-20% | 30-40% | 0-10% |
| $MC_{TC}$ | 83% | 28% | 63% | 35% | 35% | 29% | 80-90% | 60-70% | 90-100% |

*RA; Rheumatoid arthritis; Data from (Jeziorska et al., 1997, Gotis-Graham et al., 1998, Yamada et al., 2001)*

### 2.4.2 The relationship between mast cell differentiation and protease phenotype

The details of human MC differentiation and phenotypic diversification in local tissue microenvironment are not well understood. The concept about the developmental relationship between $MC_{TC}$ and $MC_T$ has been particularly controversial; whether these phenotypes represent committed subtypes deriving from two distinct circulating progenitors with irreversibly predetermined protease phenotypes, or whether they derive from a common circulating progenitor cell and represent functional states that MCs assume under the influence of the local microenvironment (Figure 6).

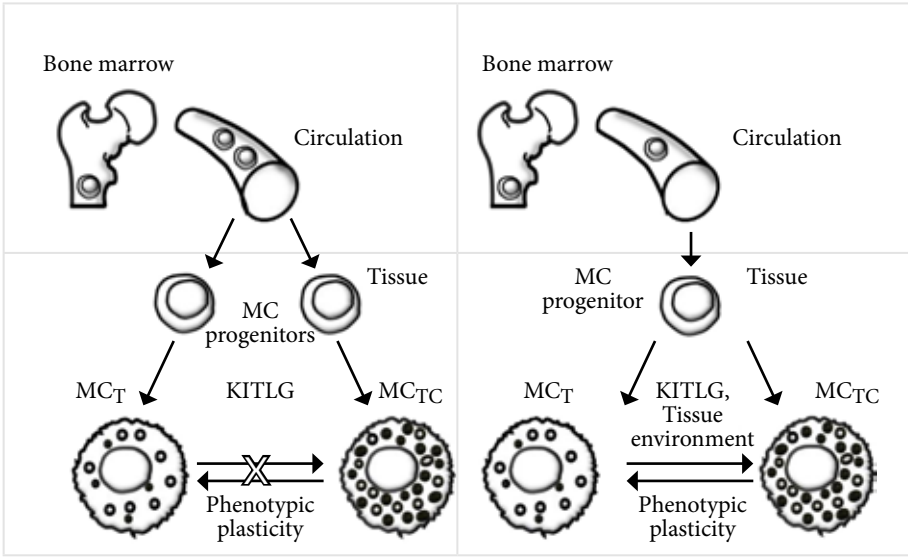



***Figure 6. Two models for human MC development.*** *MCs derive from pluripotent hematopoietic $CD34^+$ progenitor cells in the bone marrow, leave the bone marrow at their immature state and circulate as agranular $CD34^+/KIT^+$ progenitor cells. From the circulation, the MC progenitors migrate into various peripheral tissues, where they undergo terminal differentiation under the influence of local growth factors and cytokines, most importantly KITLG. Two models for the development of $MC_T$ and $MC_{TC}$ have been suggested. First, $MC_T$ and $MC_{TC}$ derive from two distinct progenitors with irreversibly predetermined protease phenotypes, and no transdifferentiation between the two MC subtypes exists (Left panel). Second, $MC_T$ and $MC_{TC}$ derive from a common progenitor cell, and microenvironmental factors, such as cytokine milieu, ultimately determine the protease phenotype. Thus, $MC_T$ and $MC_{TC}$ can switch phenotypes alongwith changes in the local microenvironment, a phenomenon referred to as phenotypic plasticity (Right panel). Adapted from (Nigrovic&Lee 2013).*

The establishment of culture systems for human MCs has provided a powerful tool for the investigation of MC development. Several factors are now known to be capable of regulating the KITLG-dependent MC development (Table 5). In human cell culture systems, T cell-derived cytokines, such as interleukin (IL)-3, IL-4, IL-6, and IL-9, promote proliferation and maturation of MCs (Kirshenbaum et al., 1992, Kinoshita et al., 1999, Matsuzawa et al., 2003, Lappalainen et al., 2007) and may skew the MC phenotype towards either the $MC_T$ or $MC_{TC}$ (Toru et al., 1998, Kinoshita et al., 1999). Indeed, *in vitro* systems have helped to demonstrate that MC protease phenotype may be directed at least to some extent by certain cytokine patterns that are present in the local microenvironment (Toru et al., 1998, Kinoshita et al., 1999, Ahn et al., 2000). Although several studies using *in vitro* generated human MCs (Saito et al., 1996, Toru et al., 1998, Kinoshita et al., 1999, Shimizu et al., 2002, Wong&Stevens 2005, Yasuda et al., 2005) have provided important insights about the relationship between MC development and protease expression, differences in the source of progenitor cells, culture conditions, analysis period, and methods used to detect the proteases have made it difficult to establish a clear concept of the developmental pathway(s) leading to the different MC subtypes.

| *Table 5. Growth factors and cytokines that regulate human MC development, proliferation, and survival* | |
|---|---|
| Growth factor/Cytokine | Function |
| IFN-γ | • Inhibits MC growth and differentiation (Kirshenbaum et al., 1998)<br>• Inhibits early progenitor cell division (Kulka&Metcalfe 2005)<br>• Promotes survival of cultured human MCs (Yanagida et al., 1996) |
| IL-3 | • Promotes development of MCs synergistically with KITLG (Kirshenbaum et al., 1992)<br>• Promotes growth of mature MCs (Gebhardt et al., 2002)<br>• Does not affect the differentiation of human MCs (Shimizu et al., 2008) |
| IL-4 | • Promotes MC maturation accompanied with increased frequency of $MC_{TC}$ phenotype (Toru et al., 1998, Ahn et al., 2000)<br>• Upregulates FcεRI expression (Toru et al., 1996, Xia et al., 1997, Iida et al., 2001)<br>• Increases mediator release (Iida et al., 2001)<br>• Inhibits MC growth during early stage of development (Nilsson et al., 1994b)<br>• Induces apoptosis of MC progenitors (Oskeritzian et al., 1999)<br>• Inhibits early KIT expression (Nilsson et al., 1994b, Kulka&Metcalfe 2005) |
| IL-6 | • Promotes MC maturation accompanied with increased frequency of $MC_{TC}$ phenotype (Kinoshita et al., 1999, Moon et al., 2003)<br>• Increases histamine content (Kinoshita et al., 1999)<br>• Reduces/inhibits apoptosis (Kambe et al., 2001) |
| IL-9 | • Increases proliferation of MC progenitors synergistically with KITLG during early stage of MC development (Matsuzawa et al., 2003, Lappalainen et al., 2007)<br>• Does not affect human MC differentiation (Lappalainen et al., 2007) |
| IL-33 | • Enhances the survival of naïve human MCs and promotes their adhesion to fibronectin (Iikura et al., 2007)<br>• Induce cytokine production in human mast cells even in the absence of stimuli of FcεRI aggregation (Iikura et al., 2007) |
| KITLG | • Promotes proliferation, differentiation, survival, and cell–cell and cell–substratum adhesion (Okayama&Kawakami 2006)<br>• Stimulates selective growth of human MCs from hematopoietic progenitors (Irani et al., 1992b, Valent et al., 1992, Mitsui et al., 1993, Kinoshita et al., 1999, Maaninka et al., 2013)<br>• Induces expression of various human MC neutral proteases (Maaninka et al., 2013)<br>• Induces production of tryptase and histamine (Valent et al., 1992)<br>• Induces chemotaxis (Nilsson et al., 1994a)<br>• Activates mediator release (Columbo et al., 1992, Sperr et al., 1993)<br>• Enhances degranulation and cytokine production through crosslinking of FcεRI (Hundley et al., 2004) |
| NGF | • Promotes development of cord blood-derived human MCs (Welker et al., 2000)<br>• Promotes MC maturation by up-regulating tryptase, FcεRI, and histamine (Welker et al., 1998)<br>• Prevents apoptosis of cord blood-derived human MCs synergistically with KITLG (Kanbe et al., 2000) |
| Neurotrophin-3 | • Promotes maturation of human intestinal MCs (Lorentz et al., 2007) |
| TGF-β | • Inhibits MC development (Kinoshita et al., 1999, Hjertson et al., 2003, Ishida et al., 2003) |

*IFN-γ, interferon-γ; NGF, nerve growth factor; TGF-β, transforming growth factor-β*

## 2.5 Mast cells and atherosclerosis

Histopathological studies of atherosclerotic lesions have emphasized the presence of increased numbers of MCs in the atherosclerotic arteries compared to the level in normal healthy arteries (Atkinson et al., 1994, Kaartinen et al., 1994b, Jeziorska et al., 1997). In normal human coronary arterial intima only few MCs (on average 1 MC/mm$^2$, 0.1% of total intimal cells) are present while in fatty streaks fivefold higher values (on average 5 MCs/mm$^2$, 0.9% of total intimal cells) have been reported (Kaartinen et al., 1994b). The number of MCs in the cap and core regions of atherosclerotic plaques in the coronary arteries is on average 2 MCs/mm$^2$ (0.5% of total intimal cells), while the shoulder regions of the plaques contain on average 6 MCs/mm$^2$ (1.1 % of total intimal cells) (Kaartinen et al., 1994b). The actual sites of plaque erosion and rupture are highly infiltrated with MCs (28 MCs/mm$^2$, 6% of total intimal cells) suggesting a role for MCs in these atherothrombotic events (Kovanen et al., 1995). In the human aorta, even normal intima contains substantial numbers of MCs (15 MCs/mm$^2$) and the numbers do not increase in fatty streaks (15 MCs/mm2) or atheromas (3 MCs/mm$^2$) (Kaartinen et al., 1994a). Thus, the number of MCs in the intima of coronary and carotid arteries, but not of aorta, increases as the atherosclerotic plaques become more advanced (Kaartinen et al., 1994a, b, Kovanen et al., 1995, Jeziorska et al., 1997). The medial layer of both normal and atherosclerotic arteries are mainly devoid of MCs (Kaartinen et al., 1994a, Jeziorska et al., 1997), although occasional medial MCs have been reported in atherosclerotic arteries (Atkinson et al., 1994). In the outermost layer of the vessel wall, the adventitia, high numbers of MCs are seen even in normal coronary arteries (19 MCs/mm$^2$), and their numbers are increased in atherosclerotic arteries both in non-ruptured plaques (41 MCs/mm$^2$) and in ruptured plaques (98 MCs/mm$^2$) (Laine et al., 1999). The accumulation of MCs in the arteries is considered to be mediated by KITLG expressed in the arterial ECs and SMCs (Miyamoto et al., 1997), and by eotaxin expressed in activated vascular SMCs (Haley et al., 2000).

In the aortic and coronary intima, all MCs contain tryptase, and a proportion of MCs also contain chymase (Kaartinen et al., 1994a). The percentage of chymase-containing MCs however, whether in aorta or coronary of healthy or atherosclerotic intima, shows high interindividual variation (Kaartinen et al., 1994b, a, Kovanen et al., 1995). MCs containing cathepsin G have also been detected in atherosclerotic arteries (Mäyränpää et al., 2006). Indeed, a recent immunohistochemical study analyzing the occurrence of MCs in atherosclerotic plaques showed significantly higher numbers of tryptase$^+$, chymase$^+$, and cathepsin G$^+$ MCs in atherosclerotic lesions compared to healthy vessels (Rohm et al., 2016). The various MC phenotypes are likely to provide different functional properties following activation/degranulation *in vivo*.

Indeed, activation/degranulation of MCs is a common feature in the atherosclerotic plaques (Kaartinen et al., 1994b, Kovanen et al., 1995, Jeziorska et al., 1997). In human coronary atheromas, the number of degranulated MCs is especially high at the shoulder region, known to be prone to rupture (Kaartinen et al., 1994b), and at the actual sites of erosion or rupture in myocardial infarction (Kovanen et al., 1995). At these sites, the proportion of degranulated MCs is approximately 85%, whereas in normal intima the levels are 17-28% (Kaartinen et al., 1994b, Kovanen et al., 1995). Accordingly, MC activation is considered to be one of the pathologic mechanisms in disease progression. Atherosclerotic plaques contain various components with potential to activate MCs however, the exact mechanisms of MC activation in atherosclerotic plaques are largely unkown. Potential activators of MCs in the context of atherosclerosis include inflammatory mediators such as oxLDL (Kelley et al., 2006), lysophosphatidic acid (Bot et al., 2013), complement anaphylatoxin C5a (el-Lati et al., 1994, Laine et al., 2002, Oksjoki et al., 2007), and mediators secreted from local macrophages and T-lymphocytes (Kaartinen et al., 1994b, Kovanen et al., 1995). Moreover, previous study (Oksaharju et al., 2009) reporting activation of MCs *in vitro* by bacteria shown to be present in atherosclerotic plaques suggests that these bacteria could act as activators of MCs in atherosclerotic lesions. In addition, the neuropeptide substance P induces MC activation (Bot et al., 2010) and is expressed by nerve fibres that co-localize with MCs in human coronary arteries (Laine et al., 2000), thus rendering neuropeptides as potential MC activators, as well. Previous studies (Kovanen et al., 1998, Wang et al., 2011) also provide evidence that one of the potential activation pathways of MCs in atherosclerotic lesions acts through FcεRI (Shi et al., 2015).

### 2.5.1 Mast cells promote formation of foam cells by proteolysing LDL and HDL

Upon activation, MCs are capable of releasing a wide variety of mediators that may contribute to atherogenesis. The increased numbers of MCs in fatty streaks (the sites of foam cell formation) and more advanced atherosclerotic plaques (Atkinson et al., 1994, Kaartinen et al., 1994b, Jeziorska et al., 1997), together with studies reporting MC activation at sites of coronary atheroma erosion or rupture (Kaartinen et al., 1994b) strongly support the contention that the MC plays an important role in atherosclerosis both in its early and late stages. During the early events of atherosclerosis, MCs may contribute to increased accumulation of LDL in intima. Histamine released from locally activated MCs is capable of increasing endothelial permeability and thus enhancing the transendothelial transport of plasma LDL into tissues (Ma&Kovanen 1997). On the other hand, heparin PGs from exocytosed rat MC granules are capable of binding to apoB-100 in LDL (Kokkonen&Kovanen 1987), thus facilitating proteolysis of apoB-100 by granule-bound chymase and CPA (Kokkonen et al., 1986). The sequential proteolysis of

apoB-100 by these rat MC neutral proteases results in fusion of LDL and the formation of larger lipid droplets on MC granules (Kokkonen&Kovanen 1989). These extracellular granule remnants carrying fused LDL can be taken up by macrophages (Kokkonen 1989, Kokkonen&Kovanen 1989) and SMCs (Wang et al., 1995) resulting in the formation of foam cells (Kaartinen et al., 1995, Wang et al., 1995). The contribution of MCs to foam cell formation is supported by the fact that in atherosclerotic lesions of carotid arteries, human MCs are observed in close association of macrophages and extracellular lipids, as well as sites of foam cell formation (Jeziorska et al., 1997).

Mast cells may promote lipid accumulation within the arterial wall also by inhibiting RCT. Indeed, MC chymase is capable of degrading apoA-I in pre-β-HDL as well as various other apolipoproteins of HDL, namely apoA-II, apoA-IV, and apoE, and thereby to generate dysfunctional HDL particles with impaired ability to induce cholesterol efflux from macrophage foam cells (Lee et al., 1992, Lindstedt et al., 1996, Lee et al., 1999, Lee et al., 2002a). The mechanism seems to include the inhibition of the ABCA1-dependent efflux pathway (Favari et al., 2004). Furthermore, also tryptase is capable of degrading apolipoproteins in HDL thus blocking its function as a cholesterol acceptor (Lee et al., 2002b). On the other hand, MC chymase and histamine are capable of inhibiting LDL oxidation (Lindstedt 1993, Lindstedt et al., 1993) indicating that activated MCs may also have antiatherogenic properties.

### 2.5.2 Mast cells contribute to plaque destabilization

The various inflammatory cells within the arterial intima are capable of secreting a large repertoire of enzymes that may degrade collagen and other components of the extracellular and pericellular matrices. Thus, macrophages may secrete proforms of MMPs, which may be activated by chymase and tryptase released from activated MCs. The inflammatory cells also secrete proinflammatory mediators capable of suppressing collagen synthesis by SMCs. Degradation of the pericellular matrix of SMCs by MC chymase may result in apoptotic death of the SMCs. These processes will, jointly or separately, decrease collagen production and increase collagen degradation within atherosclerotic plaque leading to thinning of its fibrous cap, which again renders the plaque prone to erosion and/or rupture (Kovanen 2007b).

# AIMS OF THE STUDY

Mast cells are present in human atherosclerotic lesions, and various functions for MCs in atherogenesis both in its early and advanced stages have been established. Many of these functions have been attributed to neutral proteases that are stored in high amounts within MC secretory granules and released upon activation. Within the arterial intima neutral proteases released from the activated MCs may interact with LDL and HDL, the two lipoproteins crucial in the process of atherogenesis. During atherogenesis LDL and HDL particles are subjected to various structural and compositional modifications resulting in generation of LDL particles with increased proatherogenic properties and HDL particles with impaired antiatherogenic properties, respectively. It has become obvious that such processes have adverse effects on the pathogenesis of atherosclerosis. The aim of this thesis was to identify mechanisms by which human MCs may contribute to atherogenesis focusing on HDL and LDL modification by MC neutral proteases.

Specifically, the aims were to:

1) Identify neutral proteases that are expressed by cultured human peripheral blood progenitor-derived MCs (Study I)

2) Analyze the ability of the various neutral proteases expressed by the cultured MCs to degrade the major protein components of HDL and LDL particles, namely apoA-I and apoB-100 (Study II and II)

3) Identify, whether the potential proteolysis of apoA-I affects its anti-inflammatory properties (Study II)

4) Identify, whether the potential proteolysis of apoB-100 in LDL affects the PG-binding properties of LDL (Study III)

# MATERIALS AND METHODS

| Method | Original publication |
|---|---|
| ***Cells*** | |
| Isolation of human peripheral blood-derived $CD34^+$ progenitors | I, II, III |
| Isolation of human peripheral blood monocytes | II |
| *In vitro* differentiation and culture of human mast cells | I, II, III |
| *In vitro* differentiation, culture and activation of human monocyte-derive macrophages | II |
| Activation of human mast cells | II, III |
| Culture and activation of HCAECs and THP-1 cells | II |
| Binding assays of HCAECs | II |
| Migration of THP-1 cells | II |
| ***Proteins and genes*** | |
| Immunofluorescence staining (cytospin) | I |
| Immunofluorescence staining (human coronary artery sections) | III |
| Flow cytometry | I |
| Enzyme-linked immunosorbent assays (ELISA) | I, II |
| Protease activity assays | I, II, III |
| Quantitative real-time RT-PCR | I, II |
| NF-kB translocation | II |
| ***Lipoproteins and apoA-I*** | |
| HDL/LDL isolation from human plasma | II, III |
| ApoA-I/LDL proteolysis | II, III |
| Radioactive labeling of apoA-I/LDL | II, III |
| LDL acetylation | II |
| Preparation of reconstituted HDL | II |
| Cholesterol amplex red | III |
| Proteoglycan binding | III |
| Circular dichroism | III |
| Electron microscopy | III |
| ***In vivo*** | |
| Mouse peritonitis model | II |
| ***Data*** | |
| Statistical analyses | I, II, III |

## 3 Mast cell culture and activation

### 3.1 Isolation of human peripheral blood-derived CD34$^+$ progenitors

CD34$^+$ progenitors were enriched from mononuclear cells, which again were isolated from buffy coats (concentrated leukocyte suspension) prepared by the Finnish Red Cross Blood Service (Helsinki, Finland) from peripheral blood of voluntary blood donors. The use of buffy coats in the study had been approved by the Ethics Committee of the Finnish Red Cross. The buffy coats (40 ml) were first diluted two-fold in Dulbecco's Phosphate buffered saline (DPBS) and separated into distinct phases [plasma, peripheral blood mononuclear cells (PBMCs), granulocytes, and erythrocytes] by centrifuging the buffy coat suspensions layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) at 800 × g and 4°C for 30 min. The interface layer of the PBMCs was then collected and washed three times with DPBS (first at 800 x g and 4°C for 5 min, and then twice at 250 × g and room temperature for 5 min) to remove contaminating cell types. Finally, the PBMCs were suspended in 50 ml of pre-chilled MACS buffer [DPBS supplemented with 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA)], and their total number was determined.

The CD34$^+$ progenitor cells were next enriched from the PBMCs using positive immunomagnetic selection according to the instructions of the manufacturer (Miltenyi Biotech). Briefly, the PBMCs were simultaneously incubated with human IgG (to block the Fc-receptors) and with CD34 hapten antibody (to label the cell surface antigens) at 4ºC for 15 min. The cells were washed with pre-chilled MACS buffer, and incubated with CD34 anti-hapten MicroBeads at 4ºC for 15 min. After washing with MACS buffer (at 300 × g and room temperature for 6 min), the cells were passed through MACS separation column placed in a magnetic field of the MACS separator (Miltenyi Biotech). The magnetically labeled CD34$^+$ cells were retained in the column, while the other cell types were eluted and discarded by washing the column four times with MACS buffer. The CD34$^+$ cells were eluted with 5 ml of pre-chilled MACS buffer by removing the column from the magnetic field, after which they were washed once. Finally, the number of CD34$^+$ progenitor cells was counted and the cells were resuspended in basal culture medium [Iscove's Modified Dulbecco's Medium (IMDM) with L-Glutamine and 25 mM Hepes (Biowhittaker, Lonza) supplemented with 100 U/ml penicillin (Biowhittaker, Lonza), 100 µg/ml streptomycin (Biowhittaker, Lonza), 100 µM β-mercaptoethanol (Sigma-Aldrich), 100 ng/ml recombinant human (rh)KITLG) (PeproTech, Rocky Hill, NJ, USA), and 20% serum substitute, BIT 9500 supplement (containing BSA, human recombinant insulin and human transferrin) (Stem Cell Technologies, Vancouver, British Columbia, Canada) at 0.5 x $10^6$ cells/ml. Cell sedimentation during the washing steps was performed at 300 × g and room temperature for 6 min.

## 3.2 *In vitro* differentiation and culture of human mast cells

For typical experiments, the isolated $CD34^+$ progenitor cells were grown under serum-free conditions according to a previously published protocol (Lappalainen et al., 2007). The basal culture medium was supplemented sequentially with rhIL-3 (5 ng/ml), rhIL-9 (15 ng/ml), rhIL-6 (50 ng/ml) (PeproTech, Rocky Hill, NJ, USA) as well as human low-density lipoprotein (LDL) (10 µg/protein/ml) that was isolated as previously described (Havel et al., 1955, Radding&Steinberg 1960) from the plasma of healthy volunteers supplied by the Finnish Red Cross Blood Service. Fresh culture medium was provided twice a week during the first three weeks of culture, and weekly thereafter. For the first four weeks, the cells were cultured at 37°C in a humidified incubator flushed with a mixture of 5% $O_2$, 5% $CO_2$, and 90% $N_2$ and thereafter under normoxic conditions (21% $O_2$ and 5% $CO_2$) at 37°C. Cell viability was determined weekly by Trypan blue exclusion test. The cells were cultured for a total of 9 to 10 weeks, after which the cells have been shown to express mature MC phenotype (Lappalainen et al., 2007). The cell concentration was kept around $0.5 \times 10^6$ cells/ml during the whole culture period. In separate experiments (Study I) MCs were generated by culturing the isolated $CD34^+$ progenitors with KITLG alone in the presence or absence of 10% FBS.

## 3.3 Activation of human mast cells

Mature MCs at a concentration of $2 \times 10^6$ MCs/ml were activated at week 9 of culture by incubation for 30 min with 1 µM calcium ionophore A21387 (Sigma-Aldrich) in DPBS in a humidified incubator at 37°C (21% $O_2$ and 5% $CO_2$). After incubation, the MCs were sedimented, and the supernatant containing the released neutral proteases referred to as the MC releasate was collected and stored at -80°C until further analysis.

## 4 Protease activity assays and protease inhibitors

The amounts of active neutral proteases in MC releasates were determined using specific protease substrates and/or inhibitors. Tryptase (β-tryptase) concentration in MC releasates was determined through hydrolysis of N-(p-Tosyl)-Gly-Pro-Lys-4-nitroanilide (GPK-4NA; Sigma-Aldrich, St Louis, Mo) by incubating the releasates for 60 minutes with 0.25 mM GPK-4NA in PBS containing 0.02% heparin and 0.15 mol/L NaCl at 37°C (pH 7.5) and measuring the increase in optical densities at 405 nm with a microplate reader. Recombinant human β-skin tryptase (Promega) was used to construct the standard curve. Chymase concentration in the MC releastes were determined through hydrolysis of a chymase-specific substrate, acetyl-L-Arg-Glu-Thr-Phe-4-nitroanilide (RETF-4NA), generously provided by Dr George Caughey (Veterans Affairs Medical Center, San Francisco, Calif). The substrate has been shown to be selectively hydrolyzed by chymase but not by cathepsin G (Raymond et al., 2009). For chymase determinations, MC releasates were mixed with 1 mM RETF-4NA in 0.5 mol/L Tris-HCl containing 2 mol/L NaCl and 9% dimethyl sulfoxide (pH 8.0), and changes in optical densities at 405 nm were immediately registered for 5 minutes at 30-second intervals at room temperature. Chymase activity in the releasates was then converted to a mass (μg) by using chymase-specific activity under the above-mentioned conditions. Cathepsin G levels in the releasates were determined through hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (AAPF-4NA; Sigma-Aldrich). Cleavage of the substrate by chymase was excluded by using a peptidic inhibitor, diphenylNa-benzoxycarbonyl-L-Arg-Glu-Thr-PheP-phosphonate (RETF-(OPh)2), generously provided by Dr Gunnar Pejler (Uppsala University, Uppsala, Sweden). RETF-(OPh)2 selectively inhibits chymase, while allowing full activity of cathepsin G (Raymond et al., 2003). For cathepsinG determinations, MC releasates were preincubated with 670 nM RETF-(OPh)2 in 0.1mol/L HEPES buffer (pH7.5) for 30 minutes on ice. The reaction was then initiated by the addition of AAPF-4NAat a final concentration of 1.8mM, and changes in optical densities at 405 nm were monitored for 10 minutes at 30-second intervals at room temperature. Cathepsin G activity in the MC releasates was converted to a mass (μg) by using cathepsin G–specific activity under the above-mentioned conditions. Granzyme B concentration in the releasates was determined with a commercial assay according to the instructions of the manufacturer's (Sigma-Aldrich). All concentrations were expressed as μg protease/ml releasate. In some experiments, tryptase, chymase, and CPA3 in the MC releasates were inhibited as follows: tryptase with 200 μg/ml leupeptin (Sigma-Aldrich), chymase with 670 nM (RETF-(OPh)2, and CPA3 with 1 μl/100 μl Carboxypeptidase Inhibitor from potato tuber (Sigma-Aldrich).

# 5 LDL

## 5.1 Proteolysis of LDL

Human LDL (d = 1.019–1.050 g/ml) were isolated from plasma of healthy volunteers (supplied by the Finnish Red Cross Blood Service) by sequential ultracentrifugation in the presence of 3 mM EDTA, as described previously (Havel et al., 1955, Radding&Steinberg 1960). After, isolation LDL was dialyzed against LDL buffer (150 mM NaCl, 1 mM EDTA, pH 7.4) and the protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific). LDL proteolysis was induced by incubation of LDL (1 or 2 mg/ml) with MC releasate, or alternatively with either 1.5 μg/ml recombinant human chymase (Sigma-Aldrich), 20 μg/ml recombinant human β-tryptase (a kind gift from Dr. Christian Sommerhoff), 4.5 μg/ml cathepsin G from human neutrophils (Biomol), 10 μg/ml CPA from bovine pancreas (Sigma-Aldrich), or 0.1 μg/ml granzyme B (QuickZyme Biosciences) in DPBS overnight at 37°C. In some experiments plasma of three healthy donors was pooled prior to LDL isolation. The pooled LDL (1 mg/ml) was then incubated overnight with the recombinant human chymase (1.5 μg/ml) or with cathepsin G (4.5 μg/ml) in the presence of 100 μg/ml PGs that were prepared from the intima-media of human aortas as previously described (Hurt-Camejo et al., 1990, Öörni et al., 1997).

## 5.2 Radioactive labeling of LDL

In some experiments, LDL was t-butoxycarbonyl-L-[$^3$H]methionine N-hydroxy-succinimidyl ester (Amersham Biosciences) according to the Bolton-Hunter procedure (Bolton&Hunter 1973). For that purpose, 100 μCi t-butoxycarbonyl-L-[3H]methionine N-hydroxy-succinimidyl ester ($^3$H-reagent) per 2 mg LDL was evaporated in a glass tube under Nitrogen gas. Next 2 mg/ml LDL in 0.1 M Borate (pH 8.5) was incubated with the evaporated $^3$H-reagent for 30 min on ice followed by incubation of the labeling reagent and LDL mix with 50μl/ml of 2M glycine in 0.1M borate (pH 8.5) for 5 min on ice. The $^3$H-LDL was then dialyzed against LDL buffer 3x500 ml (1x overnight) to remove the unbound labeling reagent, after which the concentration of the labeled LDL was determined by BCA Protein Assay kit. Finally, the activity of the $^3$H-LDL was determined by liquid scintillation counting. For that purpose, 10 μl of the $^3$H-LDL was mixed with 3 ml of scintillation liquid and the resultant photon emissions (following absorption by the scintillator of the energy transferred to the solvent molecules by the β-particles emitted from the $^3$H-LDL) were recorded using β-counter.

## 6 Statistical analyses

Statistical analyses were performed with the GraphPad Prism software, version 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS for Windows software, version 20 (SPSS Inc., Chicago, Illinois, USA). The nonparametric Kruskall-Wallis test with Dunn's multiple comparisons correction was used to compare the groups. Differences in continuous variables were assessed by analysis of variance or linear regression, where appropriate. $p<0.05$ was considered statistically significant. Correlations were investigated by calculation of Pearson's coefficient of correlation. The data are expressed as means ± SEM or ± SD.

# RESULTS AND DISCUSSION

## 7 Neutral protease expression in cultured human mast cells (I)

### 7.1 *In vitro* differentiation of human mast cells as a tool to study neutral protease expression (I)

Human cell culture systems are powerful tools to study protease expression in MCs and have become available after identification of factors promoting survival of MCs *in vitro*. To obtain human MCs for *in vitro* studies of protease expression three approaches are available: 1) to use human MC lines, namely HMC-1, LAD2, or LUVA (Butterfield et al., 1988, Kirshenbaum et al., 2003, Laidlaw et al., 2011); 2) to isolate MCs from human tissues, such as the lungs, intestinal mucosa, and skin (Kulka&Metcalfe 2010, Lorentz et al., 2015); and 3) to differentiate MCs *in vitro* from hematopoietic progenitor cells present in human cord blood, peripheral blood of adult subjects, bone marrow, or fetal liver (Irani et al., 1992a, Kirshenbaum et al., 1992, Mitsui et al., 1993, Saito et al., 1996, Xia et al., 1997, Toru et al., 1998, Kinoshita et al., 1999, Ahn et al., 2000, Kambe et al., 2000, Kambe et al., 2001, Shimizu et al., 2002, Moon et al., 2003, Wang et al., 2006, Lappalainen et al., 2007). Regarding option 1, the MC lines represent MCs of relatively immature differentiation stages that express proteases only at low levels (Guhl et al., 2010). Regarding option 2, only a limited number of MCs can be isolated from tissues, and additionally tissue MCs represent terminally differentiated cells, which do not usually divide in culture. Instead, differentiation of MCs *in vitro* from their progenitors ultimately yields high numbers of MCs, and allows characterization of protease expression during MC development. Thus option 3 was a method of choice when we set out to characterize protease expression in human MCs.

Couple of years prior to our study a protocol for generating human MCs *in vitro* had been developed at our laboratory (Lappalainen et al., 2007) to gradually move on from studies using rat MCs to studies using human MCs. The protocol utilizes $CD34^+$ progenitor cells present in peripheral blood as a source of MC precursors and has the advantage of resulting in generation of high numbers of functionally mature MCs in a relatively short period of time under well-defined serum-free conditions. Furthermore, buffy coats of healthy volunteers as the source of the $CD34^+$ progenitors guarantee a continuous availability of the progenitors for MC differentiation without major ethical concerns.

At the time, we began to characterize protease expression in human MCs several neutral proteases had been identified within their granules. These were chymase, CPA3,

cathepsin G, granzyme B, and tryptases derived from the *TPSAB1, TPSB2, TPSD1*, and *TPSG1* genes (Caughey 2016). However, virtually all studies that had examined protease expression during MC differentiation had focused solely on tryptase and/or chymase expression, whereas no studies of CPA3, cathepsin G, and granzyme B expression during MC development had been reported. Given the number of known human MC proteases and studies that had described the presence within human tissues of MCs with novel protease phenotype, namely tryptase$^+$ and CPA3$^+$ (Abonia et al., 2010, Dougherty et al., 2010) we hypothesized that heterogeneity of human MCs protease phenotypes could be more complex than initially suggested. Furthermore, the expression of the whole set of human MC neutral proteases had never been described within the same human MC, and we were intrigued to examine, whether these neutral proteases were expressed in the cultured MCs generated according to the protocol previously developed at our laboratory. Yet another question that we were interested in was the potential developmental relationship of the $MC_T$ and $MC_{TC}$ subtypes, a topic that had shared a vast interest among the scientific field of MC biology but which had remained a matter of dispute. To be able to best answer these questions we found it important to include all human MC neutral proteases identified until then in our study of protease expression in developing human MCs.

## 7.2 Cultured human mast cells express the whole set of studied neutral proteases (I)

We began our studies by generating human MCs from CD34$^+$ peripheral blood-derived progenitors under serum-free conditions with KITLG and sequential addition of IL-3, IL-9, and IL-6 according to the protocol previously developed at our laboratory (Lappalainen et al., 2007). At each week, a fraction of the cultured MCs was analyzed for expression of tryptase, chymase, CPA3, cathepsin G, and granzyme B at the levels of both mRNA and protein. As shown in Study I, Fig 1; middle and Fig. 2 the cultured MCs expressed transcripts for the various tryptase isoforms, namely TPSAB1/TPSB2 (α-/β1-tryptase/β2-/β3-tryptase), TPSD1 (δ-tryptase) and TPSG1 (γ-tryptase), as well as for chymase, CPA3, cathepsin G, and granzyme B. Previously, cultured human MCs had been shown to express transcripts for β-tryptase, chymase, CPA3, and cathepsin G (Saito et al., 2001). Furthermore, α- (Miller et al., 1990), δ- (Wang et al., 2002), and γ-tryptases (Wong et al., 1999) as well as granzyme B (Strik et al., 2007) had been identified at the transcriptional level in human MCs. Our study is however the first to demonstrate transcriptional expression of all the above mentioned neutral proteases in a single homogeneous population of MCs.

Protease phenotypes of cells can be studied by two major techniques: light microscopy of immunohisto-/immunocytochemically stained tissue sections/cells and flow cytometry.

Of these, flow cytometry is far more sensitive in detecting proteins (Ahn et al., 2000) and was thus a method of choice to analyze protease expression at the protein level. At the time, we initiated our study, 4 human MC phenotypes within human tissues had been described: 1) tryptase$^+$, chymase- ($MC_T$) (Irani et al, 1986), tryptase$^+$, chymase$^+$, CPA3$^+$, cathepsin G$^+$ (Irani et al. 1986), 3) tryptase-, chymase$^+$ (MCC) (Weidner et al., 1993), and 4) tryptase$^+$, CPA3$^+$, chymase- (Abonia et al., 2010; Dougherty et al., 2010) (Irani et al., 1986, Schechter et al., 1990, Irani et al., 1991, Weidner&Austen 1993, Abonia et al., 2010, Dougherty et al., 2010). Additionally, in human culture systems, the development of tryptase$^+$, chymase- MC ($MC_T$), tryptase$^+$, chymase$^+$ MC ($MC_{TC}$), and chymase$^+$, tryptase- MC (MCC) had been demonstrated (Irani et al., 1992a, Li et al., 1996, Toru et al., 1998, Kinoshita et al., 1999, Ahn et al., 2000, Shimizu et al., 2002, Lappalainen et al., 2007). Study I, Figure 1; left shows that the MCs generated in the presence of KITLG and sequentially added IL-3, IL-9, and IL-6 expressed protein for all studied neutral proteases, namely tryptase, chymase, CPA3, cathepsin G, and granzyme B. The expression was detected from the earliest week of analyses (week 1) to the time the study was terminated (week 9-10). During the early weeks of culture the percentages of protease positive MCs between the individual neutral proteases differed slightly, however the expression of the studied proteases appeared to follow a common pattern. Thus, the percentages of MCs positive for each individual protease progressively increased during early weeks of culture, and reached a plateau at week 6, at which time practically all MCs in the culture of all donors were positive for the complete panel of the studied proteases. Importantly, for each studied neutral protease a single continuous population of MCs expressing that protease at various levels (based on mean fluorescence intensity, MFI) was always observed, without any indication of discrete protease-positive and protease-negative MC subpopulations in the culture. This indicates development from CD34$^+$ peripheral blood-derived progenitors of single population of MCs with a novel tryptase$^+$, chymase$^+$, CPA3$^+$, cathepsin G$^+$, and granzyme B$^+$ phenotype.

### 7.2.1 KITLG triggers the expression of human mast cell neutral proteases (I)

Differentiation *in vitro* of MC populations mainly of the $MC_T$ subtype in the presence of KITLG alone initially led to conclusion that KITLG alone might be insufficient to promote development of the $MC_{TC}$ subtype (Irani et al., 1992b, Mitsui et al., 1993). During the following years Ahn and coworkers however demonstrated that human cord blood-derived MC progenitors developed into $MC_{TC}$ exclusively even when cultured under KITLG alone (Ahn et al., 2000). Furthermore, the study of Ahn and coworkers (Ahn et al., 2000) as well as that of Toru and coworkes (Toru et al., 1998) had provided evidence that chymase expression is somewhat cytokine dependent, which prompted us to raise a question whether the neutral protease expression by the MCs we had

differentiated depended on the cytokine combination (IL-3, IL-6, and IL-9) we had used. To investigate this possibility, the $CD34^+$ peripheral blood-derived progenitors were next cultured under KITLG alone, followed by analysis of tryptase and chymase expression by flow cytometry. Flow cytometric analysis showed that the percentage of tryptase-positive MCs of all MCs (defined as high KIT expression) at week 1 was 94% ± 2% and 96% ± 3% (n=4) when the $CD34^+$ progenitors were cultured under KITLG alone and under KITLG in the presence of IL-3, respectively. The corresponding percentage of chymase-positive MCs of all MCs at week 1 was 25% ± 12% and 60% ± 27% (n=4). At week 3, virtually all cells in both cultures were positive for tryptase and chymase (Study I, Fig. 3). Importantly, flow cytometric analyses always showed a single homogeneous population of MCs expressing tryptase and chymase at various levels but not discrete chymase-negative subpopulation, regardless of the presence or absence of the cytokines, indicating that circulating human MC progenitors have not been precommitted to develop into $MC_T$. The finding is in line with the study of Ahn and coworkers (Ahn et al., 2000) suggesting that all human MCs are capable of producing chymase, in contrast to what had previously been suggested (Irani et al., 1992a). The apparent discrepancy of the conclusions may be explained by the difference in the culture period. Indeed, human MCs, which contain tryptase but little or no chymase, are capable of upregulating their chymase expression during incubation with IL-4 and IL-6, resulting in a change of the phenotype from $MC_T$ to $MC_{TC}$ (Toru et al., 1998; Kinoshita et al., 1999). Irani and coworkers (Irani et al., 1992a) did not observe phenotypic switch of $MC_T$ to $MC_{TC}$ within 30 days, and thereby concluded that human $MC_T$ and $MC_{TC}$ derive from two distinct progenitors with irreversibly precommitted protease phenotype. However, depending on the source of the progenitor cells the phenotypic switch from $MC_T$ to $MC_{TC}$ may require an exceptionally long period of culture, even months (Kinoshita et al., 1999). Ahn and coworkers (Ahn et al., 2000) cultured cells for 8-13 weeks at which time all *in vitro* differentiated human MCs expressed chymase.

Importantly, MCs that were generated under KITLG alone also contained CPA3, cathepsin G, and granzyme B (Study I, Fig. 4), suggesting a novel role for KITLG as a common inducer of neutral protease expression in human MCs. Binding of KITLG to its receptor, KIT, recruits downstream signaling molecules including the basic-helix-loop-helix leucine zipper transcription factor called microphthalmia transcription factor (MITF) (Phung et al., 2011). Studies both in human (Lee et al., 2010) and mice (Pejler et al., 2007) provide evidence that MITF might be a key target of KITLG in the regulation of MC protease expression.

# 8 Proteolysis of apoA-I and apoB-100 by human mast cell neutral proteases (II & III)

During atherogenesis both HDL and LDL undergo various structural and compositional modifications that adversely affect their functionality. Factors capable of such modifications are various cell- and plasma-derived extracellular proteases present in the inflamed atherosclerotic plaques. The repertoire of such proteases include members of the MMP family, (Lee-Rueckert&Kovanen 2011) plasmin, kallikrein, and thrombin (Piha et al., 1995) and lysosomal cathepsins D, F, K, and S (Sukhova et al., 1998, Hakala et al., 2003, Öörni et al., 2004, Plihtari et al., 2010). Furthermore, upon activation and ensuing degranulation MCs release neutral serine proteases, among them chymase, which is capable of cleaving the various apolipoproteins present in HDL as well as apoB-100 of LDL (Kokkonen et al., 1986, Lee-Rueckert&Kovanen 2011). However, since human tissue MCs are known to contain several other neutral proteases, namely tryptase, CPA3, cathepsin G and granzyme B (Caughey 2016), which were expressed by our *in vitro* differentiated MCs, we were intrigued to study the ability of these proteases to cleave the major protein components of HDL and LDL, namely apoA-I and apoB-100, respectively.

## 8.1 Chymase and cathepsin G generate C-terminally truncated apoA-I and apoB-100 (II & III)

We began our studies by activating cultured human MCs to degranulate and thus to release their neutral granule proteases in the culture medium to obtain MC releasate for the *in vitro* studies. As shown in Study I (Fig. 1, *right panel*), the cultured MCs accumulated the various neutral proteases during their maturation at least up to 9 weeks of culture, at which point the human MCs were activated to degranulate for obtaining conditioned medium (from now on referred to as MC releasate) for the experiments. The MC releasates contained 11.2 ± 5.8 μg/ml of tryptase, 1.3 ± 0.4 μg/ml of chymase, 3.2 ± 1.2 μg/ml of cathepsin G, and 0.09 ± 0.1 μg/ml of granzyme B (n = 5 donors in duplicate experiments), as determined by activity assays used to measure the amount of enzymatically active neutral proteases released to the culture medium.

The MC releasate was then collected and incubated with LDL and lipid-free apoA-I. Treatment with MC releasate led to proteolysis of both apoA-I and apoB-100. Indeed, MC releasate induced loss of apoA-I C-terminal reactivity in a concentration-dependent manner (Study II;Figure 1C, right) as well as extensive proteolysis of apoB-100 of LDL (Study III, Figure 1A-C). To further analyze the individual contribution of each of the 5 released proteases to apoA-I and apoB-100 degradation we used specific protease inhibitors and/or individual neutral proteases. As shown in Study II, Figure I in the

online-only Data Supplement, inhibition of chymase with RETF-(OPh)2, an inhibitor that selectively inhibits chymase without affecting cathepsin G (Raymond et al., 2003), almost completely blocked apoA-I degradation, whereas inhibition of both chymase and cathepsin G completely blocked it. In contrast, inhibition of tryptase, CPA3, or granzyme B had only minor if any effect on degradation of apoA-I. The results thus indicate that among the neutral proteases released by activated human MCs, chymase is the main protease responsible for the C-terminal cleavage of apoA-I. Chymase-treatment completely depleted the full-length apoA-I and generated a large-sized polypeptide which had lost immunoreactivity against a monoclonal antibody recognizing a C-terminal epitope covering the amino acid residues 211–220. Instead, chymase did not modify immunoreactivity of apoA-I against a monoclonal antibody specific for the N-terminal region (amino acids 2–8) (Study II,Figure 1C, left), an observation in agreement with a previous report (Lee-Rueckert et al., 2008).

When we set out to characterize the proteolysis of apoB-100 of LDL by human MC neutral proteases, we had reason to believe in the destruction potential of chymase since both rat and human chymase had previously been shown to degrade apoB-100 (Kokkonen et al., 1986; Plihtari et al., 2010). Thus, were we more interested in the proteolytic activity of the other human MC neutral proteases on apoB-100. A particular interest focused on cathepsin G, due to its ability to cleave many peptide and protein targets that are also cleaved by chymase [reviewed in (Pejler et al., 2007)]. Indeed, we found that cathepsin G efficiently fragmented apoB-100 of LDL (Study III, Fig. 1 A-C). Incubation with cathepsin G yielded release from $^{3}$H-labelled LDL of TCA soluble fragments that represented 12.9 ± 0.9% of the apoB-100 of LDL, whereas incubation with chymase yielded release of 4.2 ± 0.1%, followed by tryptase (3.3 ± 0.1%), and CPA (2.5 ± 0.3%). In these conditions, granzyme B was practically unable to fragment apoB-100 of LDL (Study III, Fig 1A). When all proteases, except cathepsin G, had been inhibited with specific inhibitors, MC releasate resulted in release of about 10.7 ± 2.3% of TCA-soluble fragments, a finding in line with the results obtained with purified cathepsin G. Furthermore, treatment of LDL with cathepsin G led to almost total loss of C-terminal reactivity of apoB-100, as indicated by ELISA (Study III, Fig. 1B) that specifically detected the apoB-100 but not the C-terminally truncated apoB-48 that contains the 48% of the N-terminal sequence of apoB-100. Taken together, chymase and cathepsin G generate C-terminally truncated apoA-I and apoB-100, respectively.

## 8.2 Effects of chymase on the anti-inflammatory actions of apoA-I on endothelial cells and leukocytes

MC chymase had previously been shown to efficiently cleave lipid-free apoA-I and to deplete preβ-HDL particles, and so to block their ability to promote cholesterol efflux from macrophage foam cells *in vitro* and *in vivo* (Lee-Rueckert et al., 2011). Current data provide evidence that by regulating cellular cholesterol homeostasis, apoA-I and HDL can also regulate inflammatory responses in various cell types present within the arterial intima [reviwed in (Mineo&Shaul 2013)]. Since apoA-I and HDL have direct anti-inflammatory actions on ECs and leukocytes [reviewed in (Mineo&Shaul 2012)] both of which influence vascular health and disease, we were interested, whether cleavage of apoA-I by human MC chymase also affected its anti-inflammatory effects on these cells.

### 8.2.1 Chymase-dependent proteolysis impairs the ability of apoA-I to bind to and to suppress NF-κB-dependent proinflammatory responses in HCACs (II)

Endothelial activation plays crucial role in atherogenesis especially in its early stages by inducing expression of chemotactic factors such as VCAM-1 and ICAM-1, which recruit immune cells, mostly monocytes to arterial vessel wall (Rao et al., 2007). Both lipid free apoA-I and HDL have been shown to exert anti-inflammatory effects for activated ECs of human, bovine and murin origin *in vitro* (D'Souza et al., 2010, Van Linthout et al., 2011, Cheng et al., 2012). In rabbits acute vascular inflammation and VCAM-1 and ICAM-1 expression in the aortic endothelium are blunted by HDL *in vivo* (Patel et al., 2010), however glycated apoA-I derived from *in vitro* modification or from hyperglycemic individuals, impaired this anti-inflammatory property of apoA-I/HDL (Nobecourt et al., 2010). We began our studies by investigating whether cleavage by chymase affects the ability of apoA-I to suppress TNF-α-dependent VCAM-1 expression in human coronary artery endothelial cells (HCAECs). As shown in Study II, Fig. 1A, upregulation of VCAM-1 in TNF-α activated HCAECs was partially prevented by preincubation of the HCAECs with apoA-I (Study II, Fig. 1A). However chymase-treated apoA-I totally failed to suppress the TNF-α-dependent upregulation of VCAM-1 expression (Study II, Fig. 1B), a finding consistent with the observation of impaired anti-inflammatory action of glycated apoA-I on endothelial activation *in vivo* (Nobecourt et al., 2010). Consequently, chymase-treated apoA-I was also unable to inhibit THP-1 cells from adhering to and transmigrating across the HCAECs (Study II, Fig. 3C). Furthermore, chymase-treated apoA-I had an impaired ability to inhibit induction of COX-2, IL-6, and IL-8 expression in TNF-α activated HCAECs (Study II, Fig. 4A–D).

Previous studies have provided evidence that ABCA1 is not only involved in cholesterol metabolism, but also modulate the anti-inflammatory response of cells to apoA-I

[reviewed in (Mineo&Shaul 2012, 2013)]. In ECs, apoA-I inhibits adhesion molecule expression via interaction with ABCA1 (Prosser et al., 2012). Furthermore, the anti-inflammatory and antioxidant effects of the apoA-I mimetic peptide 5A are mediated via ABCA1 and NF-κB signaling pathways in HCAECs (Tabet et al., 2010). Thus, interaction of apoA-I to ABCA1 appears to essentially mediate anti-inflammatory actions of apoA-I in ECs. As shown in Study II, Figure 3A chymase-dependent proteolysis impaired the ability of apoA-I to attenuate activation in HCAECs of NF-κB (Study II, Figure 3A) and severely blunted (≥80%) the high-affinity binding of apoA-I to HCAECs (Study II, Figure 3B), suggesting that the C-terminal domain that was depleted by chymase, is required for binding of apoA-I to HCAECs and for subsequent inhibition of the NF-κB signaling pathway. The importance of C-terminal domain for apoA-I binding to ECs is further supported by a previous study reporting severly impaired binding of C-terminal deletion mutations of apoA-I to bovine ECs (Ohnsorg et al., 2011).

### 8.2.2 Chymase-dependent proteolysis impairs the ability of apoA-I to inhibit expression of proinflammatory genes and to induce cholesterol efflux in macrophage foam cells (II)

In addition to inhibiting EC activation, HDL/apoA-I also directly attenuates the activation of monocytes/macrophages [reviewed in (Mineo&Shaul 2012)]. In macrophages LPS induced expression of inflammatory cytokines is attenuated by apoA-I via ABCA1 (Tang et al., 2009), whereas silencing of ABCA1 abolishes such inhibitory effect of apoA-I (Yin et al., 2011). Moreover, apoA-I exerts anti-inflammatory effects on peptidoglycan polysaccharide-activated human macrophages in an ABCA1-dependent manner (Wu et al., 2014). These and other data suggest an anti-inflammatory role for ABCA1 pathway in macrophages. Considering that in macrophage foam cells ABCA1-mediated cholesterol efflux pathway is predominating (Adorni et al., 2007, Larrede et al., 2009), a dual regulatory function for ABCA1 in macrophage lipid metabolism and inflammation appears to exist (Schmitz et al., 1999). Regarding the effect of chymase-treated apoA-I on its ability to interact with macrophages, we found that chymase-treated apoA-I had impaired ability to suppress LPS-induced upregulation of TNF-α, IL-1β, IL-6, and IL-8 in human monocyte-derived macrophage foam cells of both GM-macrophage and M-macrophage subtypes (Study II, Fig. 4E-L). Furthermore, in consistent with previous studies (Favari et al., 2004, Lee-Rueckert et al., 2008) the chymase-dependent proteolysis of apoA-I also decreased the ability of apoA-I to induce cholesterol efflux from macrophage foam cells (Study II, Fig. 4M). Amino acid residues 220-231 of apoA-I have previously been shown to be necessary for functional interactions between the apoA-I and ABCA1 and thus to be required for lipid efflux *in vitro* and HDL biogenesis *in vivo* (Chroni et al., 2003). Thus, it is likely that the present finding of the decreased ability of the C-terminally truncated apoA-I generated by chymase to suppress proinflammatory response in LPS-activated

macrophages reflects impaired interaction between the truncated apoA-I and ACBA1 in the macrophages. Thus, a failure to bind to macrophage ABCA1 appears to be a molecular link between impaired anti-atherogenic effects of chymase-treated apoA-I, namely reduced anti-inflammatory and cholesterol efflux and capacities. This concept is further supported by previous findings of impaired cholesterol efflux ability and concomitant reduction in anti-inflammatory properties of oxidized (Pirillo et al., 2010) and glycated apoA-I (Hoang et al., 2007, Nobecourt et al., 2010).

### 8.2.3 Proteolysis by chymase reduces anti-inflammatory properties of apoA-I *in vivo* (II)

ApoA-I inhibits inflammatory responses also *in vivo* in experimental animals such as in mouse subjected to LPS-induced inflammation (Yan et al., 2006, Li et al., 2008). We used this well-established mouse model to examine whether chymase-dependent proteolysis would also affect the anti-inflammatory properties of lipid-free apoA-I. LPS administration remarkably increased the levels of two proinflammatory cytokines, namely TNF-α and IL-1β, whereas concurrent treatment of mice with both LPS and lipid-free apoA-I significantly reduced the level of both of these cytokines (Study II, Figure5 A&B). In contrast, concurrent treatment of mice with LPS and chymase-treated apoA-I failed to significantly reduce the levels of either TNF-α or IL-1β, indicating that the chymase-dependent proteolysis impaired the anti-inflammatory properties of apoA-I *in vivo* (Study II, Figure 5 A&B). Further studies comparing the ability of apoA-I and chymase-treated apoA-I to neutralize endotoxin activity, a mechanism by which both HDL and apoA-I have been demonstrated to exert their anti-inflammatory properties *in vivo*, suggested that impaired ability of chymase-treated apoA-I to inhibit LPS-induced inflammation resulted from a total loss of its ability to neutralize endotoxin (Study II, Figure 5C).

## 8.3 Cathepsin G and chymase generate fused LDL particles with increased PG binding affinity (III)

LDL fusion is a processes important in atherogenesis promoting both extracellular and intracellular lipid accumulation within the arterial wall (Öörni et al., 2000). Proteolysis of apoB-100 has previously been described to induce fusion of LDL particles (Paananen et al., 1995, Piha et al., 1995), which is suggested to be based on disruption of the particle integrity due to proteolysis of apoB-100 on LDL and subsequent development of unstable LDL particles prone to fuse (Kovanen&Kokkonen 1991). Apparently however, mere fragmentation of apoB-100 is not sufficient to induce LDL particle fusion. Indeed,

proteolytic degradation of LDL by trypsin (Piha et al., 1995) and α-chymotrypsin (Paananen et al., 1995, Piha et al., 1995) resulted in LDL particle fusion, whereas proteolytic fragmentation of LDL with plasmin, kallikrein, and thrombin did not (Piha et al., 1995). The inability of the latter three enzymes to induce fusion of LDL was linked to their ability to only fragment but not to release the fragments of apoB-100 from LDL (Piha et al., 1995). We found that treatment of LDL with either cathepsin G, chymase, or MC releasate, resulted in formation of enlarged LDL particles compared with native LDL (Study III, Fig 4A), and the average-sized fusion particles (29.5 ± 0.70 nm, 30.3 ± 0.60 nm, and 27.0 ± 0.32 nm for MC releasate-, cathepsin G-, and chymase-modified LDL, respectively) appeared to result from fusion of 2-3 LDL proteolyzed LDL particles (Study III, Fig. 4C). However, the largest particles that were observed were generated by cathepsin G and had diameters of about 100 nm and had thus been generated by fusion of about 100 of the averaged-sized 22-nm LDL particles.

In consistent with the previous studies showing that fusion of LDL particles results in increased affinity to PGs (Öörni et al., 1998, Hakala et al., 2001, Plihtari et al., 2010), we found that proteolysis of LDL with cathepsin G and MC releasate, and to a lesser extent with chymase, significantly increased the affinity of the LDL particles to human aortic PGs when compared to native LDL (Study III, Fig. 2A). Indeed, the number of LDL bound to PGs was almost 4-fold when LDL had been treated with cathepsin G or with MC releasate and 2-fold when LDL was treated with chymase compared to native LDL. The binding of LDL to human atherosclerotic plaques was evaluated by incubating fluorescently labeled LDL, both native and cathepsin G-treated with frozen tissue sections of human carotid plaques. We found that treatment of LDL with cathepsin G increased remarkably the binding of LDL to the plaques, as indicated by differences in fluorescence intensities (Study III, Fig. 2B & C). Furthermore, enzymatic digestion of CS caused a remarkable reduction in the binding of the cathepsin G-treated LDL (Study III, Fig. 2C & D), indicating that CS of the ECM play an important role in mediating the binding of LDL to the atherosclerotic plaques.

Potential underlying mechanisms of the increased PG-binding of the proteolyzed LDL include conformational changes in the apoB-100 secondary structure, which may had exposed cryptic domains for PG-binding (Paananen et al., 1995). Indeed, although native LDL appears to contain a single crucial PG-binding site, site B (Boren et al., 1998a), delipidated apoB-100 has been shown to contain several potential heparin-binding sites and an additional PG-binding site, designated as site A (residues 3147-3157) (Olsson et al., 1997). $PLA_2$ modification of LDL has been reported to induce conformational changes in apoB-100 rendering the site A functionally available. Consequently the site A co-operated with the site B in PG-binding resulting in increased PG-binding of the modified LDL (Flood et al., 2004). We used circular dichroism (CD) to analyze whether

proteolysis of LDL induced conformational changes in the secondary structure of apoB-100. The far ultraviolet spectra of proteolyzed LDL (Study III, Fig 3A) had similar shapes compared to native LDL but showed a slight decrease in CD intensity, indicating that proteolysis of LDL, especially with cathepsin G results in conformational changes of apoB-100. The difference between the CD spectra of cathepsin G-treated and native LDL is shown in Study III, Fig. 3B. Furthermore, it was previously reported that proteolytical fusion of LDL increases the number of basic domains of apoB-100 known to be associated with PG-binding in the fused particles (Paananen et al., 1995), providing a plausible explanation for the increased PG-binding of the proteolyzed LDL observed in the present study.

## 8.4 Mast cells are the major cathepsin G containing cell types in stable atherosclerotic lesions (III)

Classically, expression of cathepsin G has been associated with neutrophils and MCs, however based on a previous immunohistochemical study also macrophages appear to contain cathepsin G (Wang et al., 2014). We evaluated the proportion of cathepsin G containing MCs in stable atherosclerotic lesions of human coronary arteries by double immunofluorescence staining using specific antibodies against cathepsin G and tryptase. As shown in Study III, Fig. 5A-D, the majority (69 %) of the cells that stained positive for cathepsin G also stained positive for tryptase, indicating the presence of cathepsin G-containing MCs in the atherosclerotic plaques. The finding is in line with a previous study from our laboratory that reported MCs to represent on average 73% of all cathepsin G-containing cells in the coronary atherosclerotic plaques (Mäyränpää et al., 2006). In consistent with the immunofluorescence data, cathepsin G expression in the atherosclerotic plaques of coronary arteries strongly correlated with the expression of MC chymase and tryptase, and to a lesser extent with CD68, which is expressed mainly by monocyte/macrophages but also by MCs. On the contrary no correlation was found between lesional cathepsin G and neutrophil proteinase 3 (Study III, Fig. 5E-H). Taken together, MCs appear to be the major cathepsin G-containing cell type in stable atherosclerotic plaques of coronary arteries.

## LIMITATIONS OF THE STUDY

The use of neutral protease concentrations based on *in vitro* differentiated MCs may bias the relative contribution of the various neutral proteases on the determined proteolytic-dependent processes. However, we have used neutral protease concentrations that were found in the MC releasates, which reflects the ratio of the neutral protease contents within the cultured human MCs.

The use of a mouse peritonitis model weakens atherosclerosis-specific interpretation of the results. On the other hand, it provides a relevant link between *in vitro* and *in vivo* studies of the anti-inflammatory effects of chymase-treated apoA-I.

The inclusion of all three arterial wall layers in the isolation of RNA from coronaries weakens an intima-specific interpretation of the results. On the other hand the data represent the totality of inflammatory cells throughout the arterial wall layers during atherosclerosis. Another limitation is the partial degradation of RNA detected in the coronary samples. The degradation may be due to disease processes (necrosis), tissue preparation and storage such as delay in snap-freezing and the warming of the sample during intensive homogenization required for the tough consistency of the vascular tissue (Fleige&Pfaffl 2006).

Unspecific staining of the coronary and carotid samples may yield false negative or false positive results. Such stainings may be due to use of inappropriate antibodies, storage and preparation of the samples (delay before snap-feezing, inappropriate fixative), and warming of the frozen tissue while cutting. However, negative and positive controls were used to evaluate the stainings and the actual stainings were analyzed with a pathologist.

# CONCLUSIONS AND FUTURE PERSPECTIVES

Human MCs with different protease phenotypes can be found within human tissue their relative abundance changing with anatomic localizations and disease stage. Considering that activated MCs are capable of releasing high amounts of neutral proteases, the protease phenotype is likely to have a pronounced effect on the disease setting. Activated MCs are present in increased numbers in human atherosclerotic lesions, where they have potential to interact with lipoprotein particles entering the arterial intima. This thesis focused on proteolytic modifications of apoA-I and apoB-100, the major protein components of HDL and LDL particles, respectively by human MC neutral proteases, and ensuing effects on the anti-inflammatory properties of the proteolyzed apoA-I and PG-binding properties of the proteolyzed LDL.

In Study I, we describe development of cultured human MCs expressing neutral proteases chymase, CPA3, cathepsin G, granzyme B, and tryptases derived from the *TPSAB1, TPSB2, TPSD1,* and *TPSG1/PRSS31* genes. This is the first study to describe human MCs of tryptase$^+$, chymase$^+$, CPA3$^+$, cathepsin G$^+$ and granzyme B$^+$ phenotype. By showing development of a single homogeneous population of MCs with a uniform protease phenotype, and by indicating KITLG as the common inducer of the expression of all the above mentioned neutral proteases, the study suggests that human MCs have the potential to express various neutral proteases. Thus, the protease phenotype of a particular MC appears to reflect a functional state the MC has assumed under the local microenvironment, and is a subject to change along with changes in the surrounding microenvironment.

Study II identifies chymase-dependent C-terminal cleavage of apoA-I as a novel mechanism that leads to loss of its anti-inflammatory properties, being the first study to demonstrate involvement of a pathophysiologically relevant protease in the regulation of several anti-inflammatory functions of apoA-I. In study III we found a novel mechanism by which human MCs may contribute to LDL retention within the arterial wall, that is, by generating, via cathepsin G, proteolyzed LDL particles with increased PG-binding affinity, a property which is linked to the atherogeneicity of LDL. Thus, Study II and III suggest novel mechanism by which activated MCs may contribute to atherogenesis (Figure 7).

The clinical relevance of the present findings remains to be elucidated. However, since LDL retention by subendothelial PGs is an important driving force in atherogenesis (Boren&Williams 2016), determining molecules that affect LDL retention may be of clinical importance. To date LDL-C lowering drugs remains as the cornerstone

for the pharmaceutical prevention and treatment for cardiovascular disease and ongoing therapeutic improvements include even more aggressive lowering of plasma lipoproteins and treatment initiation at earlier age (Bergheanu et al., 2017). However, because accumulation of LDL within the vessel wall is recognized as a critical step in the pathogenesis of atherosclerosis, inhibition of the physical interaction between LDL and the arterial wall might offer new approaches to delay or reduce the formation of atherosclerotic lesions. As various factors are known to influence the PG-binding and retention of atherogenic lipoproteins within the arterial wall, the process offers a myriad of potential pharmaceutical targets that might lead to new therapeutic approaches against ACVD. Furthermore, since, atherosclerotic lesions contain various proteases capable of generating C-terminally truncated apoA-I, proteolytic inactivation of apoA-I is likely. Thus, regarding targeting of inflamed protease-rich atherosclerotic lesions with apoA-I, infusions of protease-resistant apoA-I mimetic peptides might be appropriate approach. Finally, better understanding of the factors that are capable of regulating MC protease phenotypes in the local microenvironments is required for a successful design of treatment strategies aimed at combatting MC-associated diseases, in which the various neutral proteases released by activated MCs contribute to the development and progression of the disease-specific pathologies.

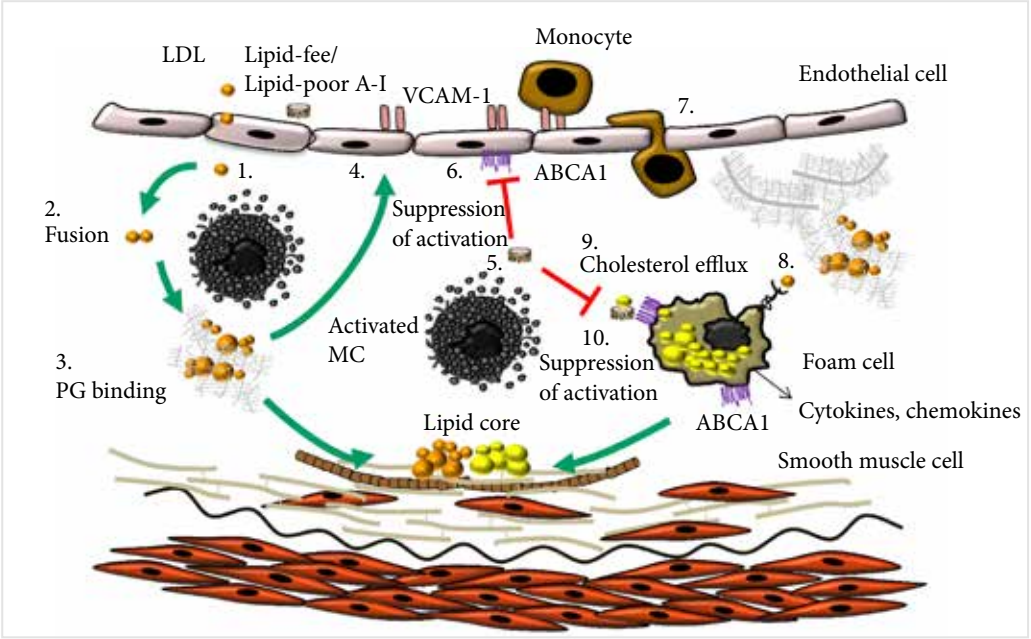



***Figure 7. Proteolysis of LDL and apoA-I within the arterial intima by neutral proteases released from activated human MCs.*** *LDL and nascent HDL/apoA-I enter the arterial intima and become proteolyzed by neutral proteases released from activated human MCs. Proteolysis of apoB-100 of LDL by cathepsin G and chymase (1) results in fusion of LDL particles generating enlarged lipid particles (2) with increased PG-binding property (3). The physical interaction between LDL and PGs hinders the exit of LDL particles from the arterial wall thus promoting intramural lipid accumulation. The retained LDL particles activate the ECs inducing expression of VCAM-1 and proinflammatory genes (4). Proteolysis by chymase generates C-terminally truncated apoA-I (5) with impaired ability of the apoA-I to bind to ECs and thus to suppress the upregulated expression of VCAM-1 and proinflammatory genes in the ECs (6). ApoA-I is also unable to inhibit the subsequent adhesion of monocytes to the activated ECs and their migration across the endothelium (7). Within the intima, the monocytes differentiate into macrophages, which uptake the retained and modified LDL particles and transform into lipid laden foam cells (8). The proteolyzed apoA-I is unable to interact with macrophage ABCA1, which results in reduced ability of the apoA-I to induce cholesterol efflux from the macrophage foam cells (9). The retained and modified LDL particles activate macrophages inducing upregulation of proinflammatory genes, a process which the proteolyzed apoA-I is unable to inhibit (10). Eventually the foam cells die contributing to formation of a necrotic lipid core. Thus, by releasing neutral proteases capable of degrading apoA-I of HDL and apoB-100 of LDL within the arterial intima, human MCs may promote lipid accumulation and inflammation, two processes critical in the pathogenesis of atherosclerosis.*

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